Interrogation of the human glutamate dehydrogenase antenna to elucidate its role in allosteric regulation and disease

by

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Dedication

This work is completely dedicated to Atlas. He was there with me when I crossed the finish line.

Acknowledgements

Thank you to my wonderful family for supporting me over the past few years. I want to thank Derek for supporting me every single day through this process and my little Atlas for giving me the strength to finish.

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Glutamate dehydrogenase (GDH) catalyzes the reversible oxidative deamination of L-glutamate using $NAD(P)^+$ as a coenzyme. GDH is found in all living organisms, but only GDH from animals is highly allosterically regulated by a wide array of metabolites. The importance of this regulation is made evident by hyperinsulinism/hyperammonemia syndrome (HI/HA), where mutations cause GDH hyperactivity. Since only allosterically regulated forms of the enzyme contain an approximately 50-residue antenna domain, it suggests that the antenna is critical for allosteric regulation of GDH. To explore the role of the antenna in allostery, a series of antenna mutations were made, including removing the antenna and characterizing clinically relevant HI/HA mutations that have been previously identified in patients. Characterization of these mutants elucidates the role of the antenna and the mechanism of GDH hyperactivity in HI/HA. Prior to this investigation, a previous antenna-less GDH construct was generated that replaced the antenna with the short loop found in bacterial GDH. The chimeric GDH lost allosteric regulation by purine nucleotides. Therefore, the purpose of the antenna was deemed to likely facilitate allosteric regulation. The current antenna-less construct, which does not include bacterial sequence, has unexpected and exciting properties as allosteric regulation

by all regulators examined is maintained. In fact, the current antenna-less construct is hypersensitive to allosteric activators ADP and leucine, but the basal activity is only about 13% that of wild-type GDH. Similarly, the characterization of the HI/HA mutants found in the antenna yielded unexpected results. It has been generalized that HI/HA-GDH hyperactivity is caused by a loss of sensitivity to GTP inhibition, but this investigation revealed that dysregulation of other allosteric regulators like hypersensitivity to activation or increased basal activity of the mutants seems to be the mechanism of hyperactivity in several mutants examined. These new insights into the antenna suggest that it plays a fundamental role not only in allosteric regulation, but in catalysis as well. Therefore, it is likely that the antenna is responsible for improving enzymatic efficiency by acting as a conduit for substrate binding energy between subunits and not solely involved in facilitating allosteric regulation.

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UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
GDH	Glutamate Dehydrogenase
hGDH	Human Glutamate Dehydrogenase
bGDH	Bovine Glutamate dehydrogenase
HI/HA	Hyperinsulinism/Hyperammonemia Syndrome
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
GTP	Guanosine-5'-triphosphate
PCA	Palmitoyl Coenzyme A
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADH	Reduced nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
75-E10	N1-[4-(2-aminopyrimidin-4-yl)phenyl]-3-(trifluoromethyl)benzene-1-sulfonamide

Chapter 1: General Introduction to Glutamate Dehydrogenase

Glutamate dehydrogenase (GDH) catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate using $NAD(P)^+$ as a coenzyme (Figure 1). Although GDH is capable of using various monocarboxylic amino acids as substrate such as L-leucine and L-alanine, its preferred substrate is L-glutamate [1, 2]. GDH is found in all six kingdoms of life, but GDH from different organisms displays distinct structural and biochemical properties and is classified by coenzyme specificity [3-5]. Its widespread distribution underscores the metabolic importance of the enzyme from bacteria, where it is involved in NH_4^+ assimilation and glutamate synthesis [6, 7], to humans and its critical role in the complex process of insulin secretion [8]. Mammalian GDH (enzyme classification 1.4.1.3) is composed of 6 identical subunits that consist of approximately 500 amino acids each (Figure 2) and is capable of using either $NADP^+$ or NAD^+ as a coenzyme. The GDH found in mammalian cells is allosterically regulated by a wide array of metabolites (Figure 3) unlike GDH from bacteria which is regulated mainly at the transcriptional level [5]. In metazoans, GDH is localized to the mitochondrial matrix where it links amino acid catabolism to glycolysis and fatty acid catabolism [8].

GDH has numerous allosteric regulator binding sites that result in a complex interplay between regulators to finely tune activity. Since product release is the rate-limiting step of GDH catalysis under most conditions [9], allosteric regulators most commonly mediate enzymatic activity by controlling this step. For example, GTP and, with ~100 fold lower affinity, ATP [8, 10] are inhibitors of the enzyme as they increase binding affinity of the product and slow enzymatic turnover. Palmitoyl-CoA is another potent inhibitor of GDH, but its mechanism of inhibition is poorly understood [11]. The confusion surrounding palmitoyl-CoA allosteric inhibition is due in large part to the fact that under certain conditions it cannot be reversed as would be expected of traditional

allosteric inhibitors like GTP. At high concentrations or long incubation times, GDH is irreversibly inactivated rather than allosterically inhibited by palmitoyl-CoA [12]. NADH is both a product of oxidative deamination and an allosteric inhibitor. It binds to a promiscuous allosteric site where it enhances the binding affinity of GTP and directly competes with ADP binding [13, 14]. ADP activation of GDH has been studied extensively and acts in a manner opposite of GTP by decreasing binding affinity of product to the active site and facilitates product release [13, 15, 16]. Leucine, in addition to being a poor substrate for GDH, is an allosteric activator. It acts akin to ADP but at a site distinct from where ADP associates [17]. Leucine and ADP are both antagonists to GTP inhibition, whereas GTP and NADH inhibit synergistically [8]. In addition to small molecules regulating activity, there are other mitochondrial enzymes that inhibit GDH such as SCHAD and SIRT4 [18-20], but it is unknown as to how these other proteins associate with GDH to modulate activity.

The extensive allosteric regulation of GDH underscores its metabolic importance. Unsurprisingly, GDH plays a broad role in human physiology including functions critical to insulin secretion, central nervous system development, and neurotransmitter homeostasis [8, 21]. In fact, mutations of GDH have been shown to be the cause of Hyperinsulinism/Hyperammonemia Syndrome (HI/HA) [22], a multi-organ disease which affects insulin secretion, CNS development, and causes increased serum ammonium levels. There is currently no treatment for HI/HA that addresses all of the symptoms of this disease by targeting GDH directly. Additionally, GDH appears to play a critical role in glutamine addicted cancer cell viability by either having increased activity [19, 23] or increased levels of expression [24]. Thus, GDH is a possible therapeutic target not only for insulin related disorders, but for certain cancers as well.

In humans and great apes, there is a second form of GDH present called GDH2. The GLUD2 gene is intron-less and likely arose from retroposition of the GLUD1 gene less than 23 million years ago onto the X chromosome [25, 26]. GDH2 expression is

found in neuronal and testicular tissues and the enzyme exhibits distinctive biochemical properties from the ubiquitously expressed GDH1. GDH2 is less sensitive to GTP inhibition, displays less thermal stability, and is much more sensitive to ADP activation than GDH1 [25]. GDH2 is also localized to the mitochondria and may play a role in CNS pathologies like Parkinson's disease [27].

GDH represents the ideal therapeutic target to treat HI/HA, stimulate insulin secretion in type II diabetic patients [28], and be used in combination with drugs that target the glycolytic pathway to treat certain cancers [23]. The only synthetic compounds that directly target GDH are not clinically useful because of low solubility, poor intestinal absorption, or off target effects. In order to identify allosteric regulators suitable for drug development and optimization, a better understanding of the critical residues in allostery and the root cause of HI/HA effects on GDH activity are required. This thesis aims to elucidate the structural basis for subunit communication that facilitates allosteric regulation by investigating key regulatory domains such as the antenna domain that is unique to allosterically regulated forms of the enzyme.

ALLOSTERY AND STRUCTURAL BASIS FOR ALLOSTERIC REGULATION OF GDH

Allostery is the process by which a ligand binds to a protein at a site distinct from the active site and elicits a change in the fundamental properties of the enzyme (i.e. changes in catalytic activity or binding affinity of a second ligand to a distal site) [29]. Allosteric regulation is common to key metabolic or signaling pathways, yet mechanisms of allostery are generally poorly understood [30]. The complex allosteric regulation of GDH has been studied for decades, but the structural mechanisms and physiological roles are still unclear.

GDH is composed of six identical subunits, which are arranged as two trimers stacked directly upon one another (Figure 2). The bottom, core domain has extensive β-

sheet interactions with the two-fold related subunit from the opposite trimer. On top of the core domains are the highly conserved NAD binding domains [31]. Metazoan GDH has a long antenna domain that rises above the NAD binding domains. The antenna from each subunit winds around the antenna from adjacent subunits within a trimer in a counter-clockwise fashion. This domain is not found in fungi, plants, bacteria, and most protists; rather, it is found only in forms of the enzyme that evolved to exhibit allosteric regulation. There is a form of GDH found in ciliates which bears a truncated antenna and limited allosteric regulation [5]. Previous studies demonstrated that removing the antenna from human GDH and replacing it with the short loop found in Clostridium symbiosum completely eliminates allosteric regulation by all regulators tested except for leucine [5].

The hexameric structure of GDH is essential for enzymatic function. Denaturation studies with increasing guanidinium chloride concentrations demonstrate that GDH is denatured in two steps [32] (Figure 4). In the first step, the two trimers are split apart and the enzyme loses activity. This is a reversible process as the trimers can reassemble into hexamers by decreasing the guanidinium chloride concentration and activity can be restored. If the guanidinium chloride concentration is further increased, the trimers break into monomers, and these monomers cannot reassemble to active enzyme. This demonstrates that monomers of GDH are unstable and inactive on their own and unable to re-associate into an active form of the enzyme. Trimers, though inactive, are stable and able to re-associate into active hexamers. Therefore, GDH must be a hexamer to be catalytically active.

Multiple crystal structures of GDH have provided insight into the dynamic nature of the enzyme [33-36]. The active site of GDH is located beneath the NAD^+ binding domain which rotates down by ~18° to clamp over the substrate bound to the active site, forming a mouth for substrate that opens and closes each catalytic cycle [31]. As the NAD binding domains rotate downward, the short, descending helix of the antenna is stretched like a spring as the longer ascending helix of the antenna rotates counter-

clockwise. The core of the enzyme expands and contracts during each catalytic turnover event [37, 38]. Known allosteric binding sites are located in points of high motion within the enzyme and likely modulate activity by controlling the motion of the protein and affecting product release, the rate limiting step of catalysis under most conditions [35, 38].

As previously mentioned, ADP activates enzyme activity by lowering the binding affinity of the product to the active site. It does this by binding at the base of the antenna behind the NAD binding domain (Figure 5). Upon ADP binding, an ion pair forms between the bound ADP and R463 (R459 of bovine GDH) of the pivot helix which likely facilitates the opening of the active site cleft promoting product release (Figure 6) [34]. When R463 is mutated to an alanine, ADP activation is eliminated, yet ADP binding is unaffected, suggesting that R463 plays an essential role in decreasing product-binding affinity irrespective of its ability to form an ion-pair with ADP [34, 37, 39].

GTP works in a manner opposite to ADP as it increases binding affinity of the product. GTP binds on top of the NAD binding domain (Figure 5). GTP appears to preferentially bind to the closed catalytic mouth conformation and stabilizes it to hinder product release (Figure 6) [31]. Due to the extensive structural information available, the mechanisms of ADP activation and GTP inhibition are well studied, but other regulators such as leucine or palmitoyl CoA are less well understood. These regulators bind to unknown binding sites and appear to act in ways that are distinct from ADP and GTP [12, 40]. Only with more structural and biochemical data can the mechanisms of these other allosteric regulators be understood.

Several cysteine residues have been implicated in in palmitoyl-CoA inhibition in studies using hGDH2, but none of these residues appears to be fully responsible for the complete inhibition of hGDH2 by palmitoyl-CoA. [11]. In this study, ten cysteine residues at 59, 93, 119, 201, 274, and 323 were mutated to alanine but only cysteine residues at positions 59, 93, 201, or 274 affected palmitoyl-CoA inhibition of hGDH2

(Figure 7). As noted earlier, palmitoyl-CoA inhibition is particularly perplexing, as it does not behave as a traditional allosteric inhibitor whose inhibition can be reversed. Therefore, all studies suggesting that palmitoyl-CoA purely acts as an allosteric inhibitor of GDH should be regarded carefully.

A large part of GDH allosteric regulation is likely mediated via the phenomena of substrate inhibition. GDH forms an abortive complex when both a product and reactant of catalysis are bound to the active site (Figure 8). In the oxidative deamination reaction and at high glutamate concentrations, 2-oxoglutarate diffuses out of the active site of GDH faster than NADH with its micromolar Kd [13, 15, 41] and is replaced with glutamate. This abortive complex has high stability and must be resolved before another catalytic cycle can begin. The allosteric activator ADP, which is known to act by lowering binding affinity of substrates and products alleviates abortive complex formation [41]. This is reflected by shifts in the substrate inhibition curves in the presence and absence of ADP (Figure 9). If glutamate levels are low, the rate-limiting step of the reaction could be glutamate binding rather than product release. Since ADP lowers binding affinity of substrate to the active site, it will inhibit catalysis under such conditions. As glutamate levels increase, the activity of GDH is stimulated by the presence of ADP. Evidence of this is clear at maximum glutamate concentration where the levels of glutamate required to inhibit the velocity is much higher in the presence of ADP. A likely explanation of this is that ADP destabilizes the abortive complex; thus, more glutamate is required to cause abortive complex formation. Although ADP regulates GDH via substrate inhibition, not all allosteric regulators show similar effects. For example, inhibitors that bind to the core of the enzyme, such as hexachlorophene, bithionol, and GW5074, do not affect this phenomenon [38, 42]. Rather, they are thought to work by slowing the enzyme via other means [38].

Another allosteric property of GDH is negative cooperativity with respect to coenzyme binding and necessarily involved inter-subunit communication. Unlike

hemoglobin, which has positive cooperativity that is typically described by the concerted (MWC, Monod-Wyman-Changeux) model [43], GDH demonstrates negative cooperativity, which can only be accounted for by the sequential (KNF, Koshland, Némethy and Filmer) model. The KNF model describes the binding of a ligand to a subunit, which induces a conformational change that in turn changes the binding affinity of adjacent subunits for the ligand in either a positively or negatively cooperative manner. Hence, the KNF model can be applied to complex systems like GDH in which there is a gradual decrease in binding affinity of a ligand rather than a single increase in binding affinity across all the subunits of the enzyme as suggested by the MWC model. In coenzyme varied steady state experiments, there are clear breaks in the Lineweaver-Burk plots representing abrupt increases in Km with increasing saturation of the enzyme (Figure 10) [38, 40]. The biological advantages of negative and positive cooperativity have been pondered for decades, and Koshland suggests that, while positive cooperativity gives an enzyme greater sensitivity over a narrow range of ligand concentrations, negative cooperativity results in a more stable enzyme activity over a broad range of substrate [43]. The structural basis for negative cooperativity in GDH is poorly understood. It was thought that the antenna might facilitate negative cooperativity between the subunits within a trimer as antenna-less forms of the enzyme, such as those found in bacteria, do not exhibit negative cooperativity, and T. thermophile GDH, which has a truncated version of the antenna, displays less pronounced changes in coenzyme binding affinity with increasing coenzyme concentrations [5]. However, more recent studies removed the antenna and observed that negative cooperativity was maintained [40]. More work must be done to fully elucidate the structural basis for negative cooperativity in GDH, but there is a clear connection between the allosteric networks involved in communicating negative cooperativity between subunits and allosteric activation by ADP. The addition of ADP has been shown to remove negative cooperativity with respect to both NAD^+ and $NADP^+$ binding to the active site [15].

Although the structural basis for negative cooperativity is unknown, there may be an energetic benefit to the enzyme by coupling coenzyme binding with reduced coenzyme affinity (i.e. the reciprocating subunit mechanism) [35]. Since product release is the rate limiting step of catalysis under most conditions, substrate binding to one subunit could prompt product release from another subunit such that catalytic turnover can continue.

GENERAL METABOLIC IMPORTANCE OF GDH IN HUMANS

The extensive allosteric regulation of metazoan GDH hints at its metabolic importance and strongly suggests that GDH does not operate at equilibrium within cells [8]. Allosteric regulation is common in enzymes that are key branch points in metabolic pathways. The allosteric regulation of GDH fine-tunes enzymatic activity to accommodate changing cellular energy needs [29, 40, 44]. In vivo, GDH is thought to operate primarily in the oxidative deamination direction as stable isotope analysis in cultured mouse islets demonstrates GDH works exclusively in this direction under normal conditions [45]. Since serum ammonium levels are usually 5000 fold lower than the Km for reductive amination [8, 15], it seems more likely that the enzyme works predominantly in the oxidative deamination reaction.

The essential role of GDH in maintaining proper insulin secretion was first suggested using a non-metabolizable analogue of leucine, BCH (β-2-aminobycyclo(2,2,1)-heptane-2-carboxylic acid) [46, 47]. Adding the activator BCH to pancreatic β-cells increased the activity of GDH, causing elevated levels of 2-oxoglutarate that then fed into the Krebs cycle. In turn, this increased the ATP/ADP ratio, and closed the ATP gated potassium channels. When the ATP gated potassium channels close, the membrane depolarizes to stimulate the influx of calcium ions through voltage gated calcium channels and results in the secretion of insulin. In an opposite manner, a

transgenic mouse model with a pancreatic β-cell-specific knockout of GDH, was shown to have glucose stimulated insulin secretion decreased by approximately 40% [48].

GDH plays a critical role in glutamate homeostasis in the CNS because glutamate is the most common excitatory neurotransmitter [49], and glutamate levels must be carefully regulated in the CNS to prevent excitotoxicity and disease [50]. As such, GDH dysregulation has been implicated in several cognitive diseases such as schizophrenia [51] and Alzheimer's [52]. In addition to maintaining glutamate-based neurotransmission, GDH also appears to play a critical role in supplementing the CNS with energy via glutamate catabolism to supplement the preferred use of glucose as an energy source. Using a GDH conditional knockout mouse in astrocytes of the CNS, Maechler and colleagues observed that, without the ability to metabolize glutamate, the brain increased glucose consumption and mobilizes energy substrates from the periphery to compensate for decreased glutamate catabolism [49]. Astrocytes, cells of the CNS that express GDH at high levels, are responsible for a variety of essential and complex functions in a healthy CNS [53], and GDH has been shown to be vital to CNS cell development and differentiation by working as an antagonist to SIRT4 [54]

SIRT4 is one of seven mammalian sirtuins that comprise a family of class III histone deacetylases, but SIRT4 does not display deacetylase activity; rather, it functions to ADP-ribosylates GDH and is localized to the mitochondria [54, 55]. The site of this ribosylation is thought to be C119 (Figure 11) [56], which is not an easily accessible residue rendering it challenging to elucidate the mechanism of modification. Nevertheless, the ADP-ribosylation by SIRT4 reduces GDH catalytic activity. SIRT4 is expressed at high levels during embryonic development and decreases in adulthood, the opposite of GDH expression levels which are high in the adult brain and nearly undetectable at embryonic day 18 in mice [54]. In a healthy fully developed CNS, the inverse expression of GDH and SIRT4 may limit the interaction of these proteins, but their relationship is still physiologically important as some cancer cells, those with a

dysfunctional mTORC1 pathway, have repressed SIRT4 [19]. In turn, repressed SIRT4 increases flux through GDH to supply the cancer cells with a source of nitrogen and metabolites for the Krebs cycle.

GDH is ubiquitously expressed throughout the body, but certain tissues such as the pancreatic β-cells, liver, kidneys, and brain express high levels of GDH. Via complex allosteric regulation, GDH can perform tissue specific roles in the CNS as a major player in glial cell development, energy substrate distribution, and glutamate homeostasis and in the pancreatic β-cells where it is critical for proper insulin secretion. The role of GDH in the liver and kidneys has long been debated as it was thought that hepatic GDH may have mediated the storage of nitrogen in glutamate by working in the reductive amination direction [57], but, with current evidence that GDH operates nearly exclusively in the oxidative deamination direction in vivo, insight into the role of GDH in the various tissues can come from examining the multi-organ disease, hyperinsulinism/hyperammonemia syndrome.

HI/HA

The physiological importance of GDH to human health was first recognized when GDH dysregulation was shown to be the cause of hyperinsulinism/hyperammonemia syndrome (HI/HA). HI/HA is the second most common form of hyperinsulinism (HI) and is typically diagnosed in early infancy [21]. Signs and symptoms of hyperinsulinism in neonates are often unrecognized and include excessive lethargy or loss of interest in feeding so children can suffer for months to years before medical intervention [58]. Diagnosis of HI is typically based on the patient's response to a closely monitored fasting test. HI/HA diagnosis requires an additional oral protein tolerance test (oPTT) and blood tests to determine serum ammonium concentrations [58]. In some cases, genetic testing is performed to confirm diagnosis or determine the specific mutation responsible for GDH dysregulation. Genetic analysis shows that there are at least 16 different residues on GDH

that, when mutated, result in HI/HA [40, 59, 60]. Since patients are heterozygous for GLUD1 mutations, it is assumed that GDH will contain a random distribution of wild type and mutant subunits within a hexamer. This assumption is based on the fact that both mutated and wild-type copies of the gene are expressed at equal levels in patients, and there is no indication that there would be any preferential association of particular subunit types that would cause anything other than a random distribution of heterohexamers. When mutated GDH is expressed using recombinant E.coli or a baculovirus expression system, a homohexameric form of the protein is produced. Mutated homohexamers lead to an exaggerated response to allosteric regulation or changes to biochemical properties compared to GDH from patient lymphoblasts heterozygous for the mutation [61].

Severe hypoglycemia can have detrimental effects to healthy brain function, as the primary energy source for the brain is glucose. During periods of fasting, the brain can utilize alternative energy sources such as ketones, but patients with HI/HA are unable to produce ketones due to excessive insulin secretion [58]. The brain is deprived of energy, which is particularly detrimental to developing brains as the developing brain's growth is a function of cerebral glucose usage [62]. Children with HI/HA often have poor neurological outcomes and can suffer from behavior disorders, learning disabilities, seizures, and speech delays [58]. Since GDH plays a large role in maintaining glutamate homeostasis in the brain, children with HI/HA have additional neurological risks. Patients with HI/HA often develop absence seizures and have an increased likelihood of developmental delays that cannot be ascribed to low glucose levels or excessive insulin secretion [54, 63].

As the name suggests, patients with HI/HA also suffer from hyperammonemia. Ammonium is a byproduct of the oxidative deamination of glutamate, so it is understandable how GDH hyperactivity can lead to high levels of ammonium being produced. There has been speculation as to what tissue is responsible for the high serum ammonium levels. The highest expression of GDH is observed in both the liver and

kidneys suggesting these organs may be the driver of hyperammonemia. Initially, a buildup of ammonia was thought to be caused by GDH hyperactivity in the liver as GDH hyperactivity depleted liver glutamate levels. Decreasing glutamate concentrations decreases the conversion of glutamate to N-acetylglutamate, a mandatory allosteric activator for the first step of the urea cycle [63]. The unlikeliness of this was later shown as therapies to treat hepatic urea cycle enzyme disorders are ineffective in treating HI/HA hyperammonemia [58]. The current consensus is that renal GDH hyperactivation causes persistently elevated ammonium levels in HI/HA patients [64]. Interestingly, patients with HI/HA do not exhibit clinical symptoms of hyperammonemia, which can include lowered appetite, poor feeding, and increased breathing rate. Although it is currently not understood why these patients are spared the side effects of hyperammonemia, alternate pathway drugs or protein restriction do not decrease ammonium levels in these patients [58].

Mutations that result in HI/HA are most often observed within the GTP binding site or the antenna domain (Figure 12). GDH hyperactivity in HI/HA is broadly thought to be caused by a loss or decrease in sensitivity to GTP inhibition. The mutations to the GTP binding site either sterically block GTP from being able to bind or disrupt bonding interactions between GTP and GDH. In the case of the well-characterized H454Y, GTP binding is blocked by the bulky tyrosine group and leads to decreased GTP sensitivity, but the basal activity of H454Y is approximately the same as wild-type GDH [61]. In response to increasing ATP concentrations, GDH generally exhibits a triphasic response. At low ATP concentrations, ATP will inhibit GDH activity by binding to the GTP binding site. As the ATP concentration increases, ATP will begin to bind to the ADP binding site and activate. If the ATP concentration continues to increase, ATP will begin to compete at the active site and inhibit once more. The ATP dose response of H454Y lacks the first phase of inhibition that is due to ATP binding to the GTP binding site [61].

The mutations to the antenna are poorly understood since there is no direct contact between the mutated residues and allosteric regulator binding sites. Several mutations to the antenna have been previously characterized using both patient lymphocytes and recombinant expression systems as sources for the mutated protein [61]. An example of an antenna mutation that is well-characterized is S448P. It is found in the disordered loop at the base of the descending helix of the antenna that connects to the pivot helix (Figure 12). Multiple studies demonstrate that the basal activity of S448P mutant is less than wild-type GDH, but that it is hypersensitive to activation by ADP [61] (Chapter 3). Another notable mutation to the antenna is P436L. P436 is positioned at the top of the short descending helix of the antenna (Figure 12). P436L is similar to S448P in that it has a lower basal activity than wild-type GDH and is hypersensitive to ADP activation (Chapter 3). What is notable about P436L is that patients with this mutation do not present with the characteristic hyperammonemia of HI/HA [65].

A rare form of HI involving GDH is Short-Chain L-3-Hydroxyacyl-CoA Dehydrogenase Hyperinsulinism (SCHAD-HI). In healthy individuals, short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) is a key player in fatty acid β oxidation [66]. Recessive mutations to the HADH gene that encode for SCHAD have been known to cause HI [67]. SCHAD is a mitochondrial protein that allosterically inhibits GDH activity via direct protein-protein interactions [68]. Mutations to the HADH gene that result in SCHAD-HI inactivate SCHAD, resulting in GDH hyperactivity as with HI/HA [68]. SCHAD-HI and GDH-HI present differently in patients. SCHAD-HI patients do not exhibit increased serum ammonium levels as in the case of GDH-HI, but uniquely have elevated blood 3-hydroxybuyrylcarnitine and urinary 3-hydroxyglutarate in some cases [58].

Both GDH-HI and SCHAD-HI fall into the category of diazoxide responsive forms of HI. Diazoxide was originally developed to treat hypertension, but, with the side effect of hyperglycemia, chronic use of diazoxide was not possible [69]. It is still under

investigation for use in emergency situations to treat hypertensive emergencies like preeclampsia and eclampsia [70]. The vasodilating effects of diazoxide are due to its ability to bind to certain KATP channels and hyperpolarize the membrane to reduce the influx of calcium ions into arterial smooth muscle cells, relaxing the cells [71].

KATP channels are not only found in the arterial smooth muscle cells, but also on the surface of pancreatic β-cells. Diazoxide binds to the SUR1 subunit of the β-cell plasma KATP channel and opens it to hyperpolarize the membrane to inhibit insulin secretion [58, 72]. Responsiveness to diazoxide is a critical distinguishing feature in children with HI, as those who are not diazoxide responsive have limited alternative drugs available to manage HI and will likely require a near-total pancreatectomy to prevent continued hyper-secretion of insulin [58]. Since introduction of diazoxide as a treatment for HI in 1964, it has remained the first line treatment for HI and the only approved treatment in developed countries [58, 73]. Additional unapproved therapies include somatostatin analogues, calcium channel blockers, and mTOR inhibitors, but these typically have limited efficacy due to a short half-life and tachyphylaxis [58].

Despite diazoxide being the first line treatment for GDH-HI and SCHAD-HI, it has a number of drawbacks. KATP channels in the peripheral tissues that contain KATP channels with SUR2 subunit are also activated by diazoxide causing many off target effects including water and salt retention that can be severe and lead to congestive heart failure, hypertrichosis (excessive hair growth), nausea and vomiting, and pulmonary hypertension [58]. The side effects of diazoxide are proportional to the dosage required to treat the patient with higher dosages being associated with more severe symptoms [58]. Additionally, diazoxide only treats hyperinsulinism and not other HI/HA symptoms. The high ammonium levels and the cognitive defects associated with HI/HA persist with diazoxide usage. Identifying an inhibitor of GDH would address major HI/HA symptoms and decrease the off-target effects of diazoxide usage.

Although GDH and its complex allosteric regulation have been studied for decades, many knowledge gaps still exist. One such gap is what is the role of the antenna in allosteric regulation. It is evident from comparing forms of GDH from different organisms that the antenna is only present in forms of the enzyme that are allosterically regulated. Previous evidence suggests that the antenna itself is the facilitator of allostery, but the work presented herein demonstrates that the antenna plays a more fundamental role to catalysis, not just allostery as was previously suggested. Additionally, antenna residues are the most frequently occurring mutations in HI/HA. These mutated residues do not contact any known regulator-binding site yet cause multi-organ pathogenesis that can be lethal if left untreated. It is currently unknown as to what role the individual antenna residues play in increasing the enzyme's activity to pathogenic levels. Since these antenna mutations are the most frequent, it is critical to understand how they affect allosteric regulation to better understand the mechanisms of HI/HA and to develop therapeutics to treat it. The work here strives to address these knowledge gaps.

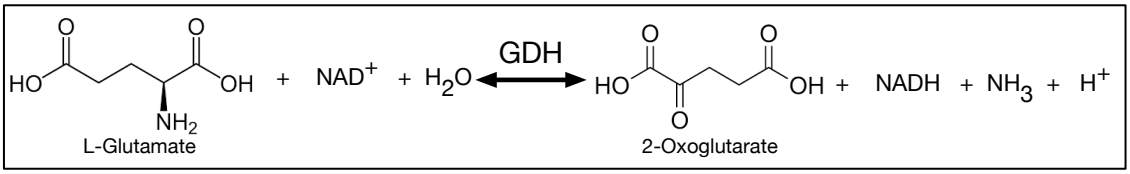

Figure 1: The reversible oxidative deamination reaction of glutamate dehydrogenase.

Although GDH from different organisms displays specificity for either NAD^+ or $NADP^+$, human GDH can use either coenzyme with equal affinity. L-glutamate is the preferred substrate of human GDH, but other amino acids such as L-leucine and L-alanine can be used with much lower affinity.

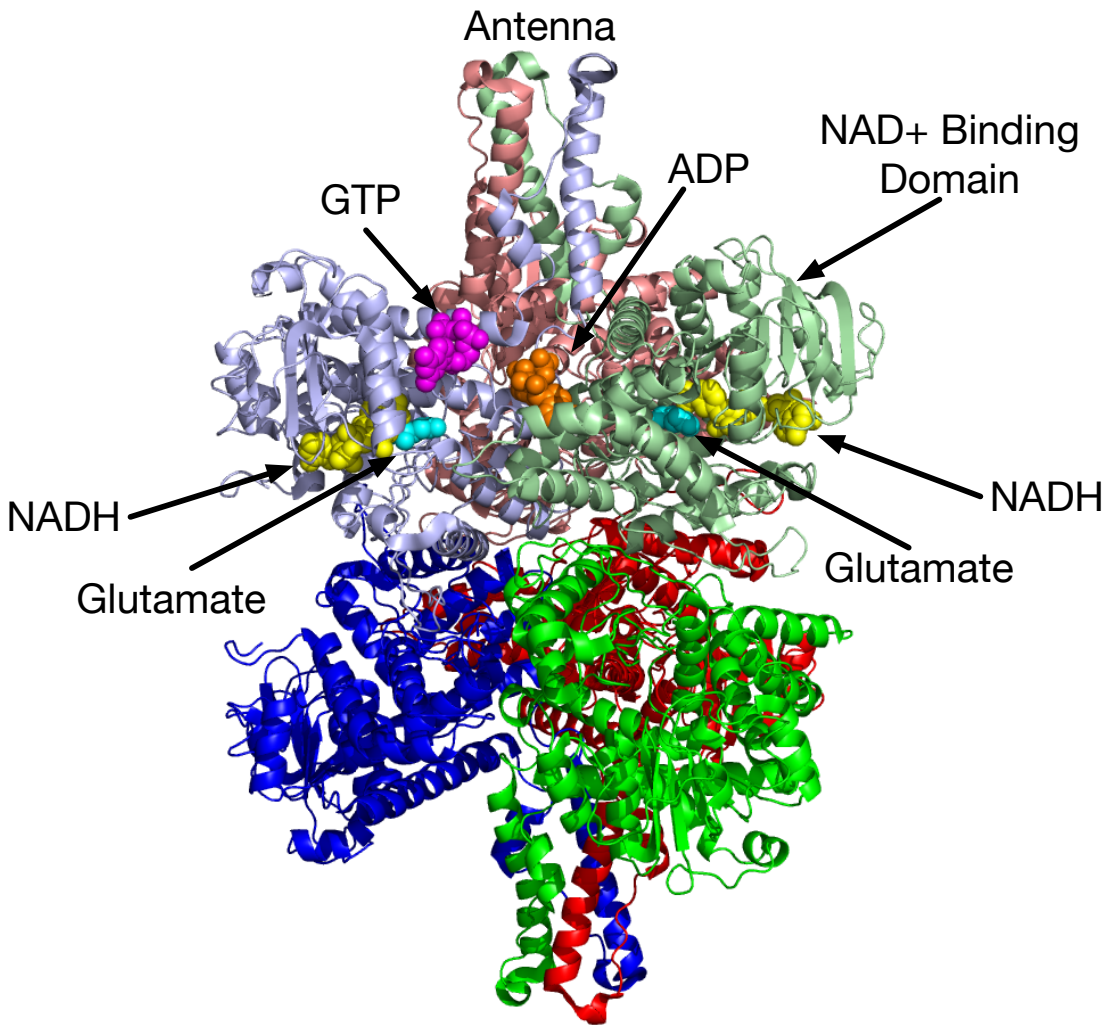

Figure 2: The structure of bovine GDH.

Each of the six identical subunits is shown in a different color for clarity. Allosteric binding sites are highlighted wih spherical models of the bound ligands. GTP is shown in magenta, and ADP is shown in orange. NADH is shown in yellow adjacent to the substrate glutamate, shown in cyan. [PDB: 1NQT and 1HWZ]

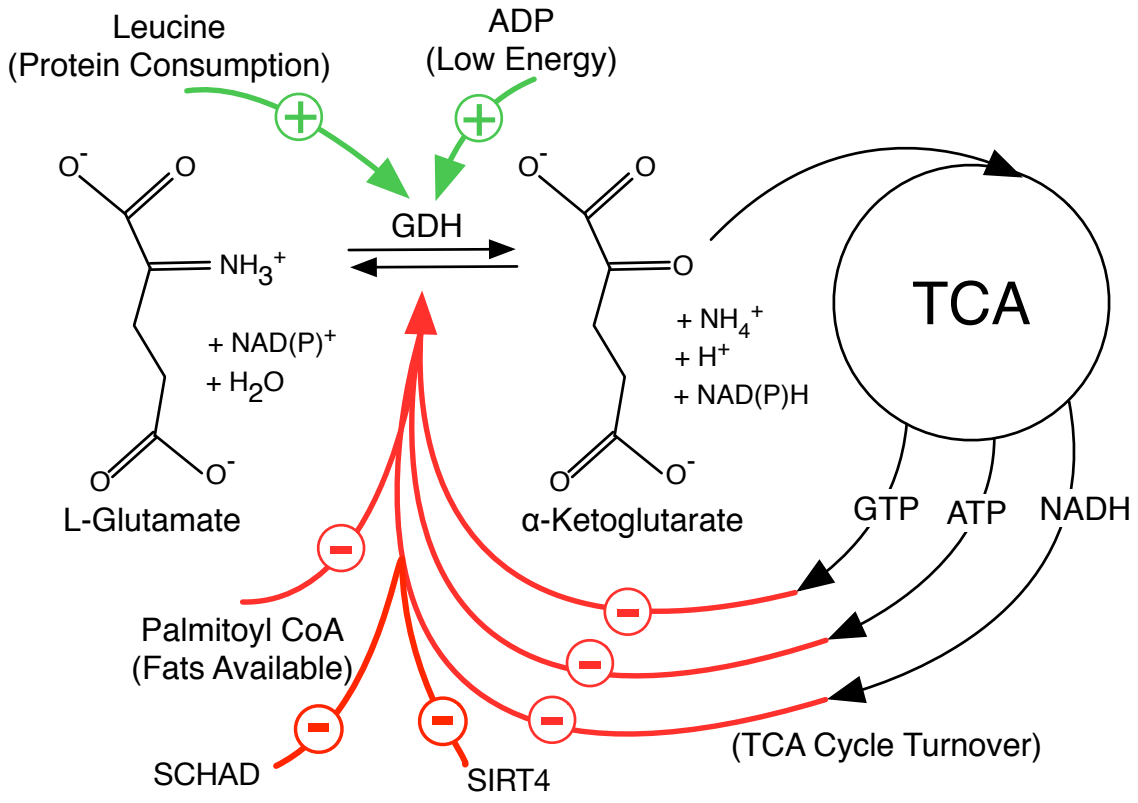

Figure 3: GDH is regulated by many allosteric regulators in vivo.

As α-ketoglutarate is fed into the citric acid cycle, allosteric inhibitors GTP, ATP, and NADH are generated. Glycolysis increases the cellular concentrations of these inhibitors making GDH sensitive to the activity of the glycolytic pathway. GDH activity is sensitive to fatty acid metabolism since GDH is inhibited by palmitoyl CoA. Thus, GDH links amino acid catabolism with fatty acid and glucose catabolism. Mitochondrial proteins SCHAD and SIRT4 are allosteric inhibitors of GDH, but their mechanism and binding sites are currently unknown. Allosteric activators in vivo include ADP and leucine. ADP levels are high when cellular energy is low. ADP stimulates amino acid catabolism via GDH to supply energy to the cell. Leucine hyperactivation of GDH by leucine is the hallmark of HI/HA.

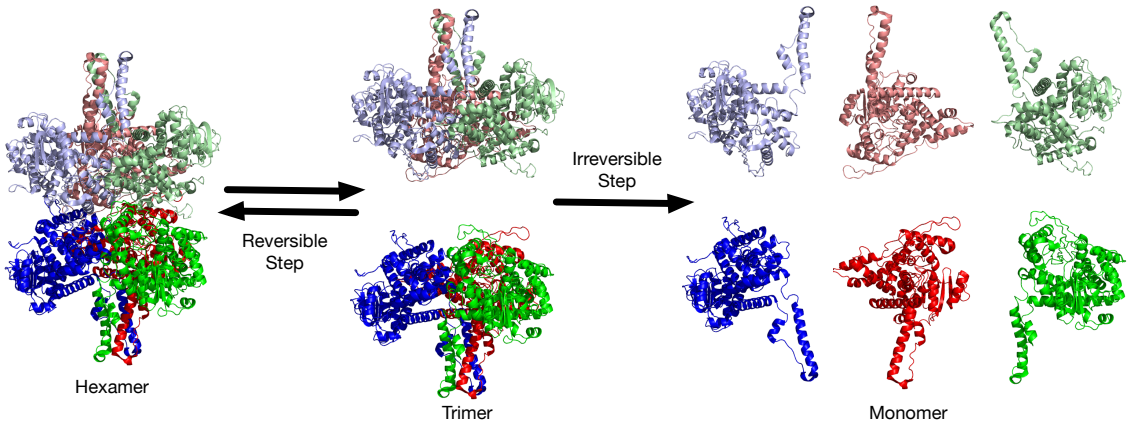

Figure 4: Steps of GDH denaturation.

GDH denaturation occurs in two steps with increasing guanidinium chloride concentrations. In the first step, a GDH hexamer is broken into two inactive trimers. This step is reversible, and the active hexamer can be reassembled by lowering the guanidinium chloride concentration. At higher guanidinium chloride concentrations, the trimers are broken into monomers. These monomers are inactive and cannot be reassembled into a trimer. It is important to note that the folded representation of the monomers is simply for clarity in this figure as it is unlikely that the monomers remain folded without the scaffolding provided by being in contact with adjacent subunits. [PDB: 1NQT]

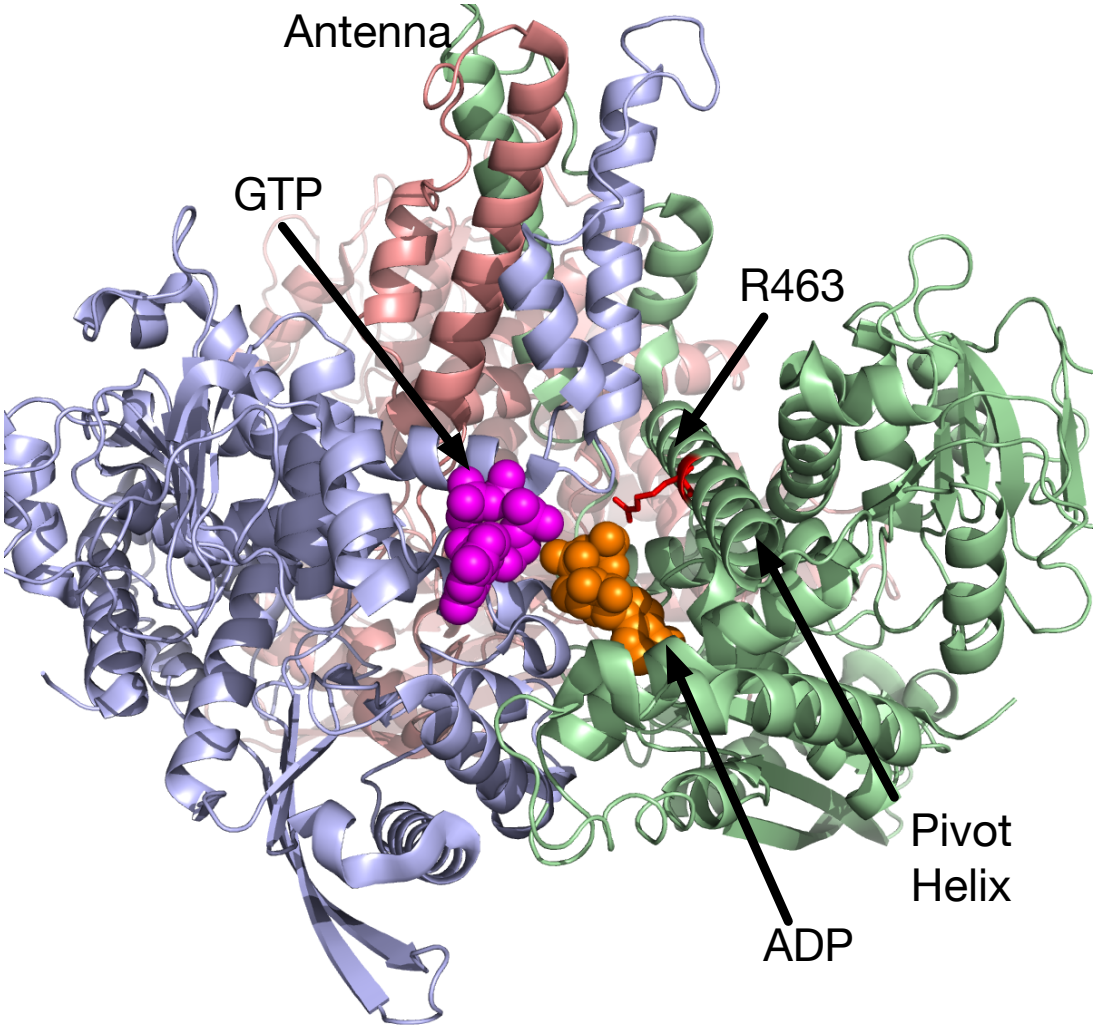

Figure 5: Cartoon representation of three GDH subunits (remaining three subunits removed for clarity).

Highlighted are the GTP (magenta) and ADP (orange) bound to their adjacent but non-overlapping binding sites. The pivot helix labeled on the green subunit contains residue R463 which forms an ion pair with the phosphates of bound ADP. This ionic interaction is crucial for activation of GDH by ADP and is thought to facilitate the opening of the mouth by decreasing the energy required for the conformational change. [PDB: 1NQT and 1HWZ]

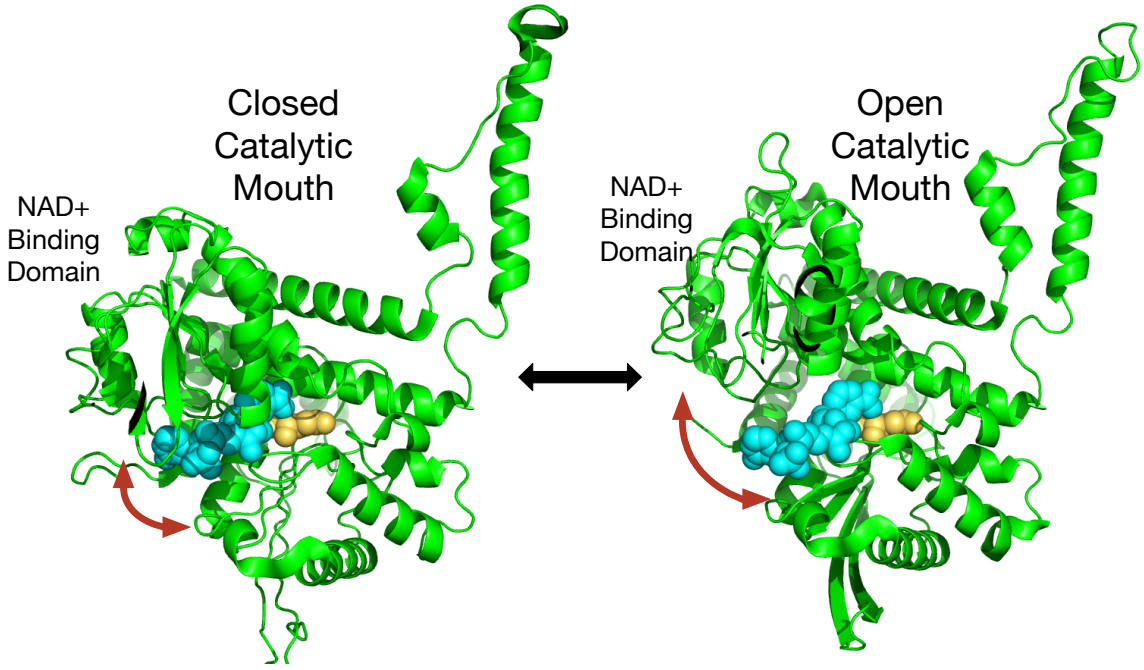

Figure 6: Diagram comparing the open and closed catalytic mouth conformational states.

GDH undergoes large conformational changes during each catalytic event. The NAD^+ binding domain rotates down by approximately 18 degrees to close over the substrate bound to the active site. Allosteric regulators make such a large transition more favorable as ADP appears to promote the opening of the catalytic mouth while GTP appears to make the closed state more favorable. Substrate is shown in cyan (coenzyme) and gold (glutamate) spheres superimposed in the active site for reference. The open and closed structures were taken from the PDB as 1NQT and 1HWZ, respectively.

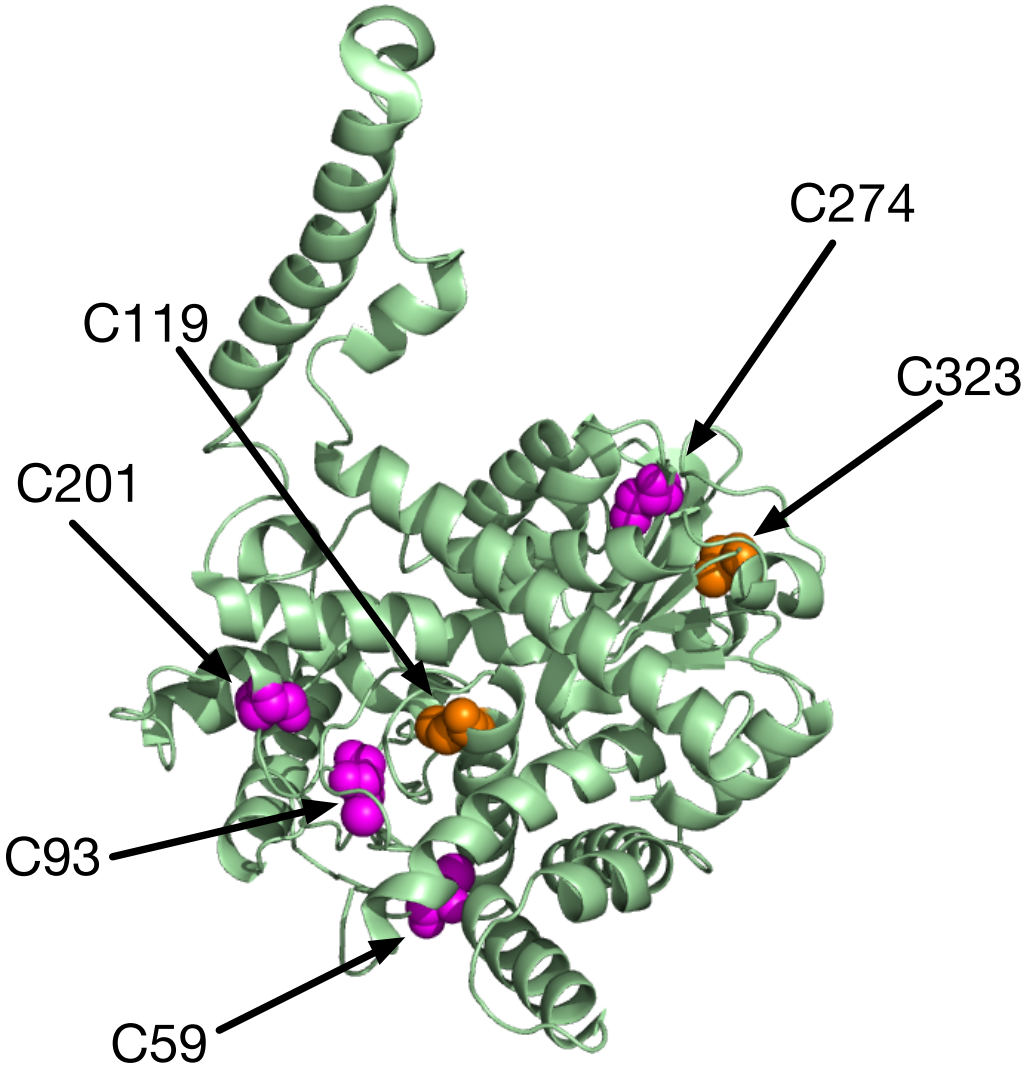

Figure 7: Cysteine residues implicated in palmitoyl-CoA inhibition.

Using site directed mutagenesis, cysteine residues were examined as potential sites of interaction with the inhibitor palmitoyl-CoA. Residues shown in magenta, C59, C93, C119, C201, and C274, when mutated to alanine, all reduced hGDH2's sensitivity to palmitoyl-CoA inhibition. Cysteine 119 and 323, shown in orange, had no effect on palmitoyl-CoA inhibition when mutated to alanine. Much work still must be done in order to elucidate any potential allosteric binding sites of palmitoyl-CoA. [PDB: 1NQT]

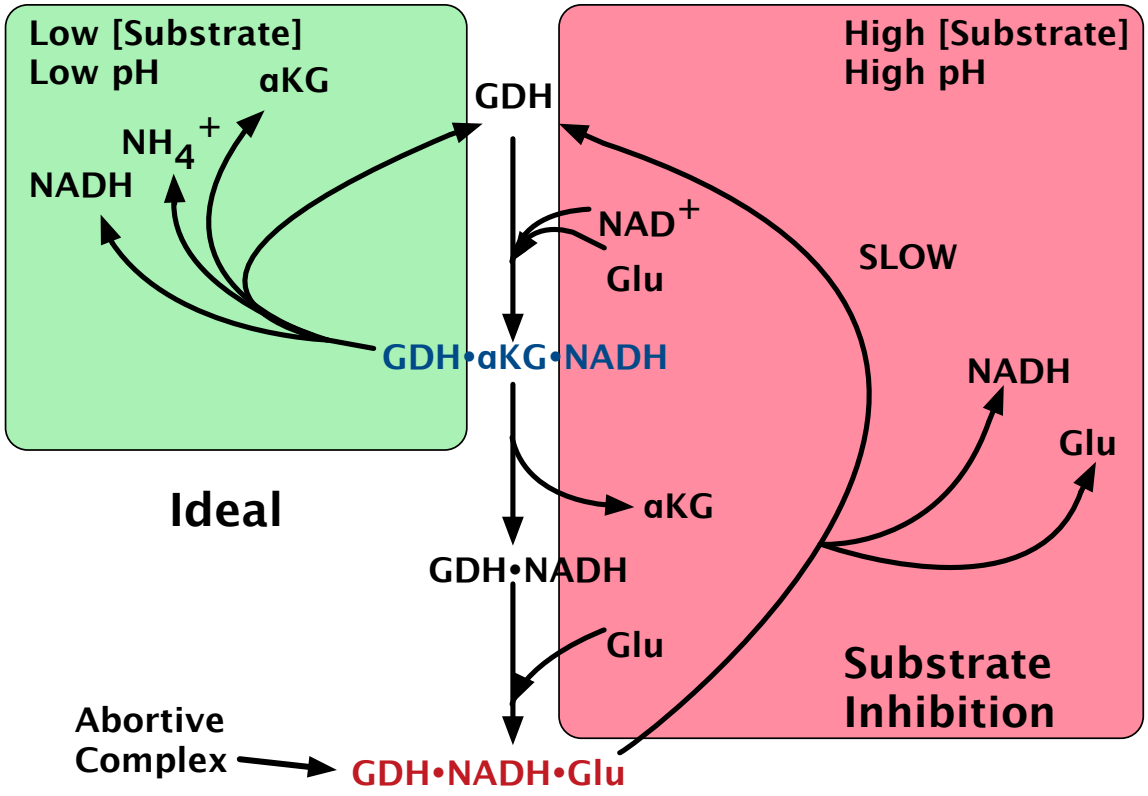

Figure 8: Schematic of abortive complex formation.

In the oxidative deamination direction, GDH binds NAD(P)$^+$ and glutamate and undergoes catalysis. Under ideal conditions, these products would be released and apo GDH begins the catalytic cycle again (shown in the green box on the left side of figure). However, the binding affinity of ammonium is lower than α-ketoglutarate which is lower than NAD(P)H. Therefore, at high glutamate concentrations, glutamate replaces 2-oxoglutarate before NAD(P)H can disassociate resulting in a tightly bound abortive complex (red box on right). Allosteric regulators GTP and ADP stabilize and destabilize the abortive complex respectively, modulating catalytic turnover rate [8].

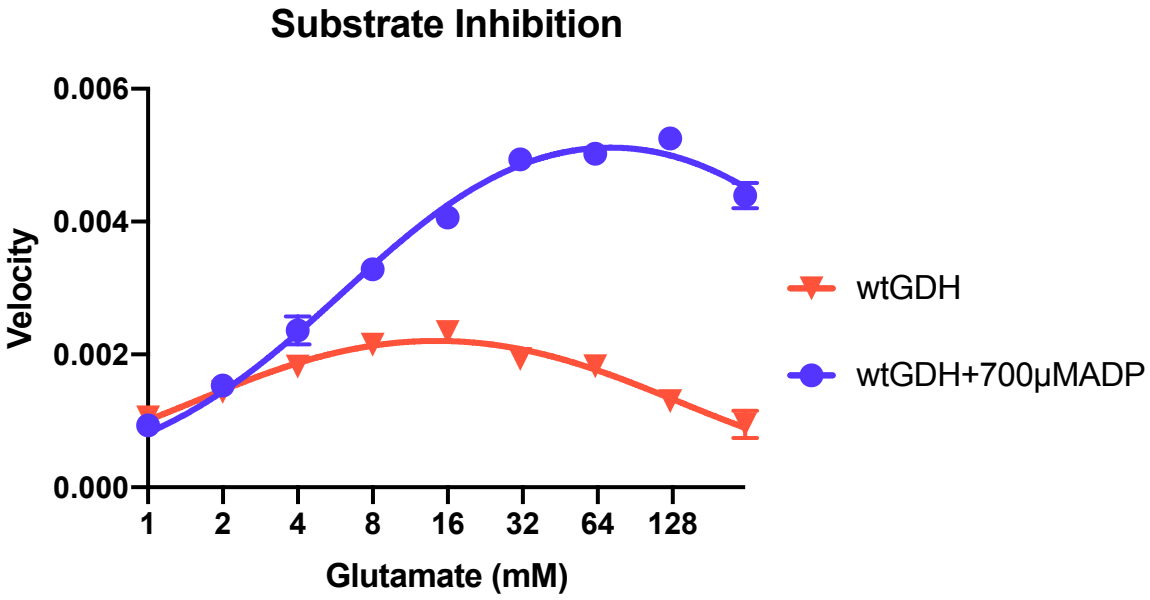

Figure 9: Substrate inhibition with increasing glutamate concentrations.

With increasing glutamate concentrations, the velocity of GDH increases initially as is expected. When substrate inhibition occurs, there is a critical concentration of substrate, which begins to inhibit the reaction velocity due to the formation of abortive complex. Since ADP has been shown to destabilize abortive complex, the addition of ADP increases the critical glutamate concentration.

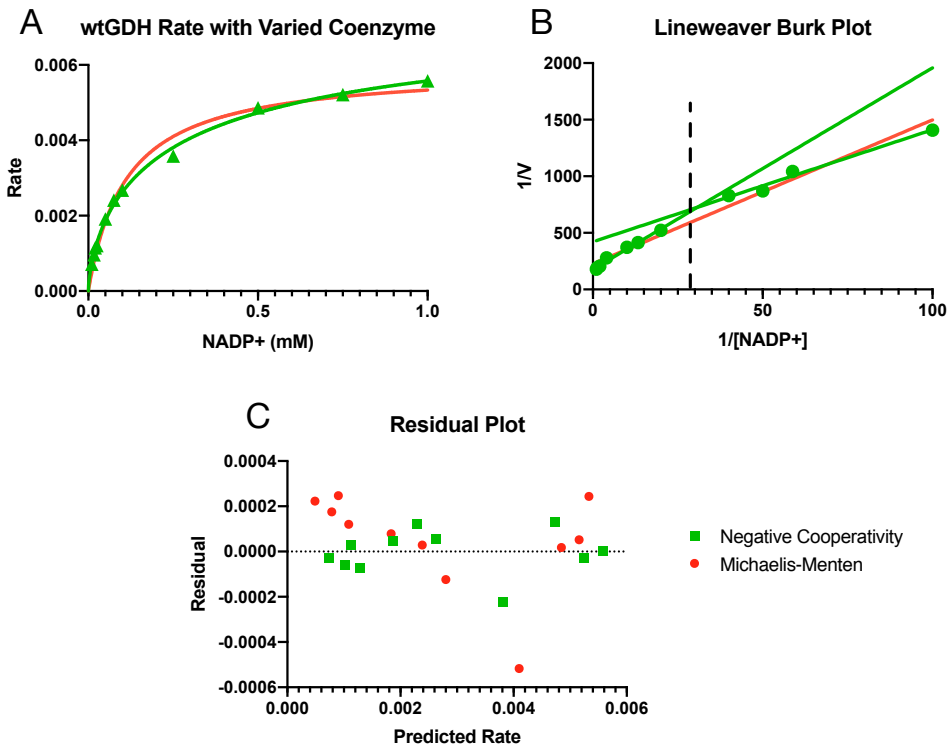

Figure 10: Negative cooperativity of GDH with respect to $NADP^+$ binding.

A. Catalytic rate of wtGDH vs. coenzyme concentration. Shown in green is the fitting of the curve assuming negative cooperativity while the red curve assumes no cooperativity. The calculated hill coefficient is 0.68, strongly indicating negatively cooperative behavior. B. The Lineweaver-Burk plot of this data also demonstrates negative cooperativity. The slope of the Lineweaver-Burk plot is inversely proportional to the Km of coenzyme. There are evident breaks in the slope of the line (shown by the dashed vertical line) indicating changes in Km (shown by green lines). The poorly fitted red line assumes no cooperativity. C. The residuals of part A show that assuming negative cooperativity produces random residuals that are centered about 0. Using Michaelis-Menten fitting produces a skewed "V-shaped" curve indicating a poor fit.

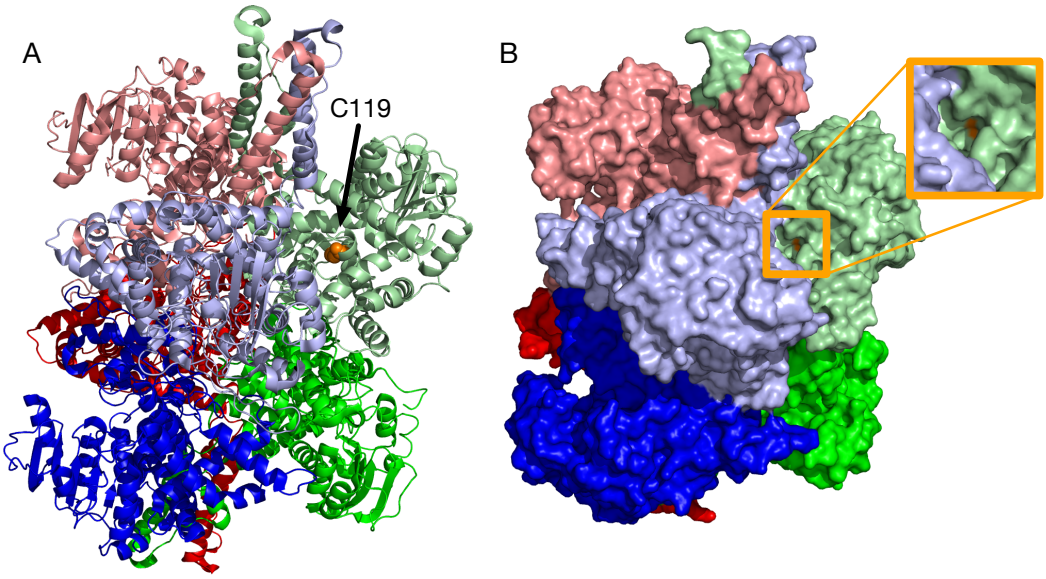

Figure 11: Location of cysteine implicated in ADP-ribosylation.

A. Cartoon diagram of the GDH hexamer with the predicted site of ADP-ribosylation highlighted in orange. The cartoon representation shows how C119 is poorly accessible to the putative ribosylation. B. A surface representation of the protein illustrates how inaccessible C119 is to proteins that would modify the residue. [PDB: 1NQT]

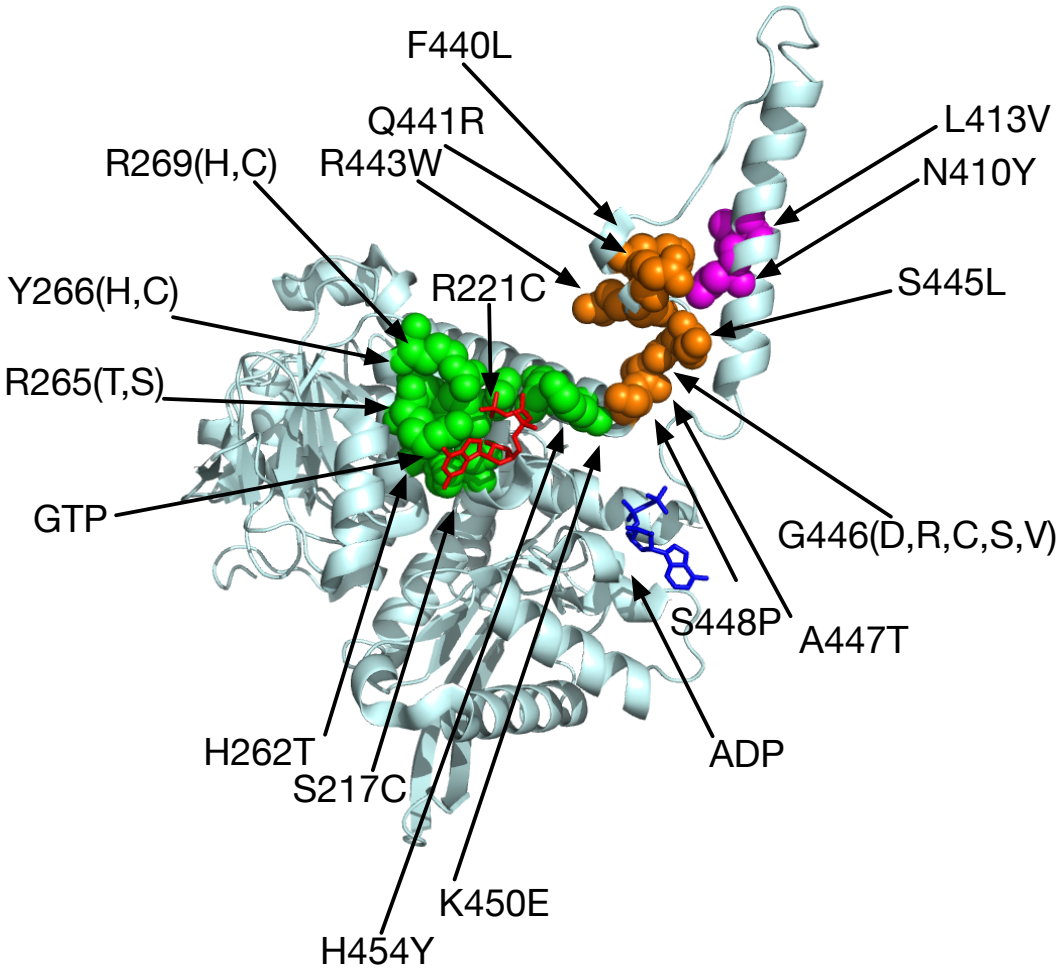

Figure 12: GDH mutations that cause HI/HA.

GDH mutations that cause HI/HA are clustered in the GTP binding site (GTP binding site residues shown in green). These modifications prevent or decrease GTP binding to GDH and subsequently prevent the enzyme's inhibition. Interestingly, the most frequently occurring mutations are in the descending helix of the antenna of GDH (residues shown as orange spheres). These and ascending helix mutations (shown as magenta spheres) do not contact any known regulator-binding site, so it is currently unknown if or how they block GTP binding. [PDB: 1NQT and 1HWZ]

Chapter 2: Dissecting the Antenna in Human Glutamate Dehydrogenase: Understanding Its Role in Subunit Communication and Allosteric Regulation

Zoe A. Hoffpauir, Eleena Sherman, and Thomas J. Smith

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ABSTRACT

Glutamate dehydrogenase (GDH) is a homohexameric enzyme that catalyzes the reversible oxidative deamination of L-glutamate. While GDH is found in all living organisms, only that from animals is highly allosterically regulated by a wide array of metabolites. Since only animal GDH has a 50-residue antenna domain, we hypothesized that it was critical for allostery. To this end, we previously replaced the antenna with the loop found in bacteria, and the resulting chimera was no longer regulated by purine nucleotides. Hence, it seemed logical that the purpose of the antenna is to exert the subunit communication necessary for heterotrophic allosteric regulation.

Here we revisit the antenna deletion studies by retaining ten more of the hGDH residues and without adding the bacterial loop. Unexpectedly, the results were profoundly different than before. The basal activity of the mutant is only ~13% that of wild type but ~100 times more sensitive to all allosteric activators. In contrast, the mutant is still affected by all of the tested inhibitors to approximately the same degree. The resulting antenna-less mutant retained its negative cooperativity with respect to coenzyme, again suggesting that inter-subunit communication is intact. Finally, the mutant still exhibits substrate inhibition, albeit there are differences in the details.

We present a model where the majority of the antenna is not directly involved in allosteric regulation per se but rather may be responsible for improving enzymatic efficiency by acting as a conduit for substrate binding energy between subunits.

INTRODUCTION

Glutamate dehydrogenase (GDH) catalyzes the reversible oxidative deamination of L-to 2-oxoglutarate using $NAD(P)^+$ as a coenzyme [74]. Although GDH is found in every organism, only animal GDH exhibits complex allosteric regulation. Animal GDH is allosterically regulated by a wide array of ligands [2, 10, 14, 15, 46, 75, 76]. GTP [13, 14, 77], and with ~100-fold lower affinity, ATP [10], is a potent inhibitor of the reaction that acts by increasing the binding affinity for the product, thereby decreasing enzymatic turnover [13]. Palmitoyl CoA [78], steroid hormones [79], and diethylstilbestrol [76] (DES) are also potent inhibitors. ADP is an activator of GDH [10, 13, 15, 16, 77] that acts in an opposite manner to GTP by facilitating product release. Leucine is a poor substrate for GDH and an allosteric activator for the enzyme [2]. Its activation is akin to ADP but acts at a site distinct from ADP [17].

Structural and sequence comparisons of GDH across the various kingdoms has shown that while the major domains and the catalytic site are conserved, animal GDH possess a unique 50-residue antenna-like feature [33-36, 80-83]. A slightly truncated form of the antenna first appears in the Ciliates concomitant with the appearance of allosteric activation by ADP and inhibition by palmitoyl CoA and may be correlated with movement of β-oxidation of fatty acids from peroxisomes to the mitochondria [5]. Subsequent evolution to animals resulted in further extension of the antenna and regulation of several other metabolites including the important inhibitor, GTP, and activator, leucine. The importance and role of this subsequently evolved regulation was made evident by the discovery of the hyperinsulinemia/ hyperammonemia syndrome (HHS) that is caused by loss of GTP inhibition of GDH [84-86]. This disorder

demonstrates that GDH plays a critical role in insulin homeostasis and this is mediated by allosteric regulation.

Therefore, the link between the antenna and allosteric regulation is clear. However, the molecular mechanism of how allostery is exacted via the antenna is not. The role of the antenna has been previously explored by removing the antenna of human GDH and replacing it with the short loop found in Clostridium symbiosum GDH [5]. This chimeric GDH lost sensitivity to regulation by ADP, GTP, and Palmatoyl CoA [5]. Further, a number of HHS mutations in GDH desensitize it to GTP inhibition but lie in the antenna, well away from the GTP binding site [21]. Indeed, the most frequently occurring mutations are located in the antenna of GDH [87].

There is an additional isoform of GDH, GDH2, found in humans and great apes, that is encoded by the GLUD2 gene, which is specifically expressed in the neural and testicular tissues. This intron-less form of the GDH gene was likely retro-posed onto the X-chromosome less than 23 million years ago [88, 89]. GDH2 is identical to GDH1 in all but 15 residues and has markedly different thermal stability and regulation [90]. Notably, Arg443, located in the descending helix of the antenna, is mutated to a serine in GDH2. This amino acid substitution was shown to increase sensitivity of the enzyme to ADP and leucine activation as compared to GDH1 [91], further evidence of the importance of the antenna to allosteric regulation.

The overall structure and areas of interest in this study are reviewed in Figure 13. Figure 13A shows the homohexameric structure of bovine GDH with the activator, ADP, bound [34] and each subunit a different color. The antenna area deleted in this study is highlighted in brown. Figure 13B shows the sequence alignments of the antenna domain

of the various forms of animal GDH. R400 (hGDH sequence numbering) is highlighted in yellow and is conserved among all naturally occurring forms of GDH. The black arrows denote the sites of some of the mutations that cause HHS by increasing GDH activity. Note that tetrahymena GDH (tGDH) has an antenna but it is shorter by seven residues on the descending strand. In our previous study [5], the focus was on converting hGDH to a chimeric hGDH/bacterial form. To this end, the entire antenna domain was deleted and replaced by the WTAEE loop found in Clostridium symbiosum. The residues deleted in the work presented here are colored brown in the hGDH1 sequence. Ten more hGDH1 residues are included in the antenna-less mutant and the bacterial WTAEE loop was not included. While hGDH2 is nearly identical to hGDH1, there are two residues that change in this region compared to hGDH1, M415L and R443S, which are highlighted in red. Figure 13C is a stereo image of the area immediately around the ADP binding site. The start and end of the antenna deletion (398-442) are noted as is the conserved R400 residue that interacts with the phosphates on ADP.

The work presented here describes a new antenna-less construct of GDH1 where the focus was not to recapitulate bacterial GDH in this region, but rather to try to retain more of the natural human GDH sequence at the base of the antenna that have been identified as HHS lesion sites. This mutant behaves markedly different than the previous antenna-less construct. This new antenna deletion greatly decreases the basal activity of the enzyme but has little to no effect on all of the allosteric inhibitors tested. In large part, the differences in allosteric regulation between this antenna-less construct and the previous hGDH/bacterial chimera [5] has to do with residues between the GTP and ADP binding sites. However, stimulation by all of the activators is greatly exaggerated in spite

of binding to different sites on the enzyme. To explain these results, a model is presented whereby the purpose of the antenna is to decrease the energy of opening and closing the catalytic cleft. With a higher basal activity, wild type GDH is more responsive to both allosteric stimulation and inhibition, making the enzyme better suited as a control point for the flux of glutamate to the Krebs cycle.

RESULTS

Role of the antenna deletion in GTP and ADP regulation

It was necessary to first assess what effect removing the antenna had on hGDH activity. To this end, enzymatic activities of pure bGDH and partially purified hGDH and antenna-less hGDH were measured. These same samples were then analyzed with Western blots (Figure 14) and the relative amounts of enzyme were estimated using the ImageJ software [92]. Using the ratio of enzymatic activity divided by the area under the curves of the bands in the Western blots, the specific activities of the samples were compared. The specific activity of hGDH was within the margin of error of bGDH. However, the activity of antenna-less hGDH was only ~13% that of wt hGDH. While it was possible that this lower specific activity is due to damaged enzyme in the antenna-less sample, it should be noted that the activity of antenna-less GDH in the presence of ADP was comparable to wt GDH in the absence of ADP (see details below). Therefore, the basal activity of antenna-less GDH is only ~1/10 that of wt GDH.

In the previous study, where the hGDH antenna was replaced with the loop found in bacterial GDH (Figure 13), there was complete loss of GTP inhibition and ADP activation [5]. Therefore, GTP and ADP regulation was first tested on this slightly more

modest antenna deletion mutant. As shown in Figure 15A and Table 2, the baculovirus-expressed wt hGDH was slightly more responsive to GTP inhibition with a slightly lower Kinh. The antenna-less GDH was slightly less responsive to GTP with about a two-fold higher Kinh and ~15% lower maximum inhibition. To verify that the effects on GTP inhibition was independent of reaction direction, the assay was also performed in the oxidative deamination reaction (Figure 15B). As with the reductive amination reaction, GTP was still able to inhibit the reaction without significant differences compared to wt hGDH. In contrast, the antenna-less GDH is far more responsive to ADP activation (Figure 15C). While both bGDH and wt hGDH shows the typical ~2-fold activation by ADP with similar Kact, the antenna-less form is activated ~10-fold. Similar to GTP inhibition, the kinetic binding constant (Kact) for ADP is ~3-fold higher in the antenna-less mutant. Therefore, just configuring the antenna deletion to contain ten residues more of the original hGDH antenna has a profound effect on purine regulation. More importantly, these results show that the vast majority of the antenna is not responsible for purine regulation.

Role of the antenna in negative cooperativity

It has been well documented that mammalian GDH exhibits negative cooperativity with respect to coenzyme binding. Using steady state kinetics, this is observable in the oxidative deamination reaction using either NAD^+ or $NADP^+$ as coenzyme [93, 94] and is observed in binding assays with NAD(P)(H) [93, 95-97]. Homotrophic negative cooperativity is indicative of subunit communication. Since the antenna within the trimers extensively intertwine, and the antenna-less forms of GDH do

not exhibit negative cooperativity, it was possible that the antenna is responsible for negative cooperativity. To test for this, the oxidative deamination reaction was measured at varied $NADP^+$ concentrations (Figure 16). As has been observed previously, [93, 94], the steady state reaction does not follow Michaelis-Menton kinetics and the data needed to be fitted with equation 3. Also shown in the insets are the same data graphed as Lineweaver-Burke plots that have been traditionally used to demonstrate the 'break' in the linearized plots indicative of negative cooperativity. bGDH, wt hGDH, and antenna-less GDH all exhibit clear negative cooperativity with Hill coefficients well below 1.0. This implies that the subunit communication associated with negative cooperativity may be mediated by the other conformational changes associated with the opening and closing of the catalytic cleft [98].

The role of the antenna in substrate inhibition

Mammalian GDH exhibits marked substrate inhibition at high glutamate concentrations in the oxidative deamination reaction at pH 8.0 and at high 2-oxoglutarate concentrations in the reductive amination reaction at pH 6.0 [15]. To ascertain whether the deletion of the antenna had any effect on substrate inhibition, the oxidative deamination reaction was examined at varied glutamate concentrations and at pH 8.0 in the presence and absence of ADP (Figure 17). As shown in Figure 17A, rather than exhibiting the normal saturation velocity curve, the velocity decreases at higher concentrations. For comparison, the concentration at which the velocity starts to decrease is noted on the curves. The datasets were fitted to equation 4 and the results summarized in Table 2. Both bGDH and wt hGDH have similar Km's for glutamate and similar

substrate inhibition constants. Unexpectedly, when the antenna is removed, the Km and substrate inhibition constants increase ~10-fold compared to wt. Together, this suggests that removing the antenna decreases the affinity of glutamate and this, in turn, alleviates substrate inhibition and increases the concentration at which it occurs. As was previously shown [15, 41], ADP shifts the inhibition curve to higher concentrations (Figure 17B), consistent with an increase in both Km and the substrate inhibition constant (Table 2). Interestingly, wt hGDH + ADP is very similar to the antenna-less hGDH without ADP. As shown in Figure 15, antenna-less GDH is hypersensitive to ADP (Figure 15B). Unexpectedly, there is an apparent reversal in the effects of ADP on the antenna-less hGDH. When ADP is added, the Km and substrate inhibition constant decreases to approximately the same levels as wt hGDH. This is rather contradictory to wild type GDH where ADP activates by decreasing substrate/product binding.

Effect of deleting the antenna on other regulators

Leucine activates GDH at a site distinct from ADP [17] and may be binding near the synthetic inhibitor, bithionol, binding site (Figure 13 [38, 99]). In addition, our recently discovered activator, 75-E10, appears to bind to the ADP site but the details of activation differ [41]. Therefore, it was necessary to determine whether removing the antenna increased the potency of all activators or just ADP. As shown in Figure 18, both activators are more efficacious with the antenna-less mutant than either bGDH or wt hGDH. Therefore, the antenna-less form of GDH is hypersensitive to all activators, no matter where they bind.

Since GTP inhibition is essentially unaffected by removing the antenna, it was then necessary to determine whether any of the other inhibitors were similarly unaffected. The inhibitor, bithionol, does not bind to the GTP site but rather binds deep within the hexamer (Figure 13 [38]). Since this mode of inhibition is independent of the GTP site, it was necessary to see if deleting the antenna had any effect on inhibition. As shown in Figure 18, bithionol inhibits antenna-less GDH nearly as effectively as wt. Similarly, EGCG, that inhibits by binding to the ADP site [37], also inhibits the antenna-less mutant.

The effects of the R400Q mutation

These results differ from the previous antenna deletion experiments where a slightly larger portion of the antenna was deleted and replaced with the short loop found in bacteria [5]. In that construct, ADP, EGCG, and GTP regulation was eliminated whereas none of these regulators are affected in the current construct. There are two likely causes for this difference. There are a number of basic residues in hGDH that are replaced by three glutamate residues in the previous construct (Figure 13). This change in charge could affect ADP and GTP binding but could also affect the conformational changes associated with regulation and catalysis. Alternatively, simply converting the antenna to such a short loop could also affect conformational changes associated with allosteric regulation.

To try to explain the differences between the previous bacterial/animal GDH chimera [5] and the truncated antenna presented here, a R400Q mutation was made in the current antenna-less mutant. The reason this site was chosen was that, as shown in Figure

13, residue 400 is a conserved residue even in tetrahymena GDH. Further, it lies immediately adjacent to the β-phosphate of the bound ADP [34].

This R400Q mutation of the antenna-less mutant had rather unexpected effects on allostery. As shown in Figure 19A, the R400Q mutation did not affect GTP inhibition. However, while the antenna-less mutant is hypersensitive to both ADP and leucine activation, the R400Q mutation eliminated activation by both (Figures 19B and 19D). ADP and leucine are believed to bind to different sites (Figure 13 [38]) and act synergistically with each other [17]. Therefore, the mutation is affecting something intrinsic to activation rather than a particular allosteric site.

Since R400 is immediately adjacent to the ADP site, we then wanted to see if the R400Q mutation had any effect on ADP antagonism of GTP inhibition. Interestingly, the R400Q mutation eliminates ADP activation alone (Figure 19B) but does not block ADP abrogation of GTP inhibition (Figure 19C). Therefore, the R400Q mutation must not be blocking ADP binding, but rather how it is activating the enzyme independent of GTP inhibition. This is very much akin to our previous R463A mutation on the pivot helix (Figure 13) that blocked ADP activation but did not affect ADP binding [34]. In contrast, the R400Q mutation blocks leucine activation in the presence or absence of GTP inhibition. This suggests that leucine only/mainly acts by activating the enzyme and not by removing GTP effects.

DISCUSSION

The role of individual antenna residues in allosteric regulation

A key difference between the previous antenna-less mutant [5] and the one presented here is the inclusion of residues at the base of the antenna. A number of these

residues have been implicated in HHS; R443, S445, G446, A447, and S448. Analysis of GDH from lymphoblast homogenates of HHS patients revealed that S445L increases the IC50 of GTP 3 to 6 times without affecting the IC50 of ADP [59]. Similarly, G446A causes a nearly 7-fold increase in the IC50 of GTP while doubling the IC50 of ADP [59]. Expression of various HHS mutants in 293T cells revealed that R443W, S445L, and G446C all increase the IC50 of GTP 600 to 800-fold [100]. The discrepancies between patient and heterologously expressed GDH mutants is due to the fact that the patient GDH is composed of both wild type and HHS subunits [61].

Interestingly, a number of these HHS mutants lie on the descending helix of the antenna. This helix appears to hyper-extend when the mouth is closed and then recoil when the mouth is open [33]. In this way, this helix might act like a spring that tends to pull the mouth open. The HHS mutants in the helix are all predicted to stabilize the helix and we suggested that such conformational changes during catalysis are critical for exacting allostery as well [33].

In addition to the HHS mutants, GDH2 also has changes in this region concomitant with alterations in allosteric regulation. Compared to wild type GDH, and similar to the antenna-less mutant, GDH2 has a markedly lower basal activity and increased sensitivity to ADP. One of the differences between GDH1 and GDH2 is that R443 is a serine in GDH2. R443 lies in the middle of the descending helix and makes a π-cation interaction with Y405 of an adjacent subunit. The R443S change in GDH2 increases sensitivity to ADP activation without affecting GTP [101]. While R443 is not mutated to serine in this antenna-less construct, it is relatively close to the NAD^+ binding domains and its location is drastically altered when the antenna is removed. Perhaps the

most important commonality between GDH2 and this construct is that they both have alterations in this same helix.

Role of the antenna in heterotrophic allostery

Removal of the antenna greatly reduced the basal activity of GDH that is restored by the addition of any one of the activators. Our current model is that the purpose of the antenna is to lower the energy required to open and close the catalytic cleft. Therefore, when the antenna is removed, the energy required to move the coenzyme binding domain increases, and the catalytic turnover rate slows.

While ADP, leucine, and 75-E10 bind to different sites or activate in slightly different ways, the antenna-less mutant is hyper-reactive to all of them. We propose that this is accomplished by increasing the energy required to move the coenzyme binding domain in a manner akin to GTP inhibition. GTP can inhibit the reaction by more than 95%. In the absence of inhibitors, these activators stimulate the reaction at most 2-fold. However, when these activators are added to the GTP inhibited enzyme, the apparent activation can be more than order of magnitude. In a similar manner, removing the antenna may create a permanently 'GTP-inhibited-like' state. This would explain the apparent hyper-stimulation by all of the activators regardless of binding sites or mode of action.

This model is also consistent with the fact that all of the inhibitors still inhibit the antenna-less mutant. If the GDH is made less efficient by deleting the antenna, it is by virtue of changing the energetics of the conformational changes associated with catalysis

rather than via a particular allosteric site. Therefore, the residual activity is still sensitive to allosteric regulation in an additive manner.

The antenna-less GDH still exhibits substrate inhibition, however, requires almost ten times more glutamate than wt (Figure 17). This is also consistent with the hypothesis that the antenna decreases the energy required to open and close the catalytic mouth. In the structure of the GDH-glutamate-NAD(P)H abortive complex, the NAD^+ binding domain is tightly closed upon the mixed substrates [35]. If the lack of an antenna makes it harder to close the catalytic cleft, then it follows that it would require more glutamate to observe substrate inhibition.

The antenna and negative cooperativity.

Mammalian GDH exhibits strong negative cooperativity with respect to coenzyme [93, 95-97]. Since this phenomenon has not been observed with the antenna-less forms found in other kingdoms, we initially surmised that the antenna was involved in the inter-subunit communication necessary for negative cooperativity [5]. However, the data presented here clearly shows that removal of the antenna does not affect negative cooperativity, showing that other inter-subunit contacts are responsible for negative cooperativity.

Effects of the R400Q mutation

The effects of the R400Q mutation on ADP stimulation strongly suggests that ADP has two separate modes of activation; direct activation of catalysis and antagonism

with GTP. The R400Q mutation blocks both ADP and leucine activation in the absence of GTP, in spite of these activators binding to distal parts of the enzyme. Therefore, ADP and leucine have some intrinsic effect on improving the catalytic turnover of GDH that involves the area around the base of the antenna. It is likely that ADP and leucine bind and activate synergistically through this first mode of activation. With ADP, the R400Q mutation does not affect abrogation of GTP inhibition and therefore cannot be blocking ADP binding. In this way, the R400Q mutation is akin to our previous R463A mutation on the pivot helix where ADP was able to bind but not activate [34].

The second mode of ADP activation appears to be abrogation of GTP binding and/or inhibition. Bound ADP and GTP are only separated by a small loop that contains S397 (Figure 13) that interacts with the β-phosphate of bound ADP [102]. When S397 is mutated to isoleucine, both ADP activation and GTP inhibition are lost [37]. It is therefore not difficult to envision how ADP binding could affect GTP binding via this same loop. In contrast, leucine presumably binds near the bithionol site (Figure 13 [99]) and does not have this same effect on GTP. From all of these results, it is very clear that this region around the ADP/GTP site junction, at the base of the antenna, is extremely important for allostery and that ADP activation is a 2-phase process.

The role of the antenna

With the caveat that residues at the base of the antenna (e.g. R400 and S397) appear to be involved in many aspects of allostery, these studies have shown what processes the majority of the antenna is not involved in. Without the antenna, all of the inhibitors tested are still functional in spite of binding to more than three separate sites on

the enzyme. Similarly, the antenna is not necessary for activation by all known activators tested. In fact, the rather sluggish antenna-less enzyme is hyper-activated by natural and synthetic compounds. Finally, and somewhat surprisingly, the antenna is not involved in the process of negative cooperativity. These results beg the question as to why nature evolved this rather unusual 50-residue feature.

We previously suggested that GDH is an energy sensor in the mitochondria (for a review, see [8]). In the presence of carbohydrates or lipids, GDH is strongly inhibited by GTP or palmitoyl-CoA, respectively. As these energy sources become depleted, the level of ADP rises and GDH begins to catabolize glutamate. Therefore, GDH needs to react to the changing energy state like a tunable rheostat rather than a simple on/off switch and the antenna helps facilitate this. Without the antenna, the enzyme has extremely low activity and therefore the addition of inhibitors does not have significant impact on the flux of glutamate to 2-oxoglutarate. Essentially, the antenna-less form can be accelerated but not braked. GDH is clearly at a crucial metabolic branchpoint and, as such, needs to have sufficient enzymatic efficiency so that it can be easily activated or inhibited depending upon the metabolic state of the mitochondria. The antenna may have evolved to this end. Indeed, it was previously suggested that GDH has a 'reciprocating subunit' mechanism whereby the energy of substrate binding to one subunit facilitates product release from other subunits [103] and this would certainly make the enzyme more efficient. Such a process could be mediated by the helix in the descending strand of the antenna that hyper-extends when the mouth is closed and recoils when the mouth opens [33]. This could be one way that the energy of substrate binding can be conserved to facilitate product release.

These results also suggest that GDH allostery is mediated by altering the energetics of this dynamic enzyme rather than locking it into a particular state. Many allosteric systems have been described as an equilibrium between R (high affinity) and T (low affinity) [104]. GDH does not fit into this simplistic model for a number of reasons. Firstly, this model is based on ligand binding to a high affinity state, driving the structural equilibrium away from a low affinity state. This explains positive cooperativity but cannot be used to model the negative cooperativity observed with GDH. Secondly, heterotrophic allosteric regulation of GDH cannot be described as a transition between an activated and inhibited forms of GDH. The most obvious example of this is ADP activation where activation or inhibition depends on environmental conditions [15]. In other words, ADP has a particular effect on the enzyme (decreasing substrate/product binding affinity) but how this translates to changes in catalytic turnover depends on the rate limiting step under those particular conditions.

Instead of R/T transitions, GDH regulation appears to be mediated by changes in the energy required to open and close the catalytic cleft. During each catalytic cycle, the coenzyme binding domain must close over the bound substrates, dehydrate the active site, and then hydride transfer can proceed. The cleft must then re-open and release product. Under most conditions, product release is the rate limiting step and this gives rise to an initial burst phase in pre-steady state kinetics [105]. Using deuterated glutamate, an isotope effect is observed on the steady state rate but not on the initial burst rate. This suggests that conformational changes are rate limiting prior to hydride transfer [9]. This is consistent with the structural model of catalysis reviewed above. It will be interesting to see whether these conformational changes prior to hydride transfer (e.g. cleft closing)

are affected by removing the antenna or adding various inhibitors (e.g. hexachlorophene) that bind to the core of the enzyme.

Finally, it should be noted that the antenna may have additional functions in-vivo that are not observed in-vitro. The inner mitochondrial matrix is essentially a protein gel. It is known that GDH associates with, and is regulated by, other mitochondrial proteins such as short chain 3-hydroxylacyl-CoA dehydrogenase (SCHAD) [68] and SIRT4 [18, 54]. It may be that the antenna also plays some role in assembling multi-enzyme, regulatory complexes within the mitochondria. Clearly, further studies are necessary to better understand the structural basis of the complex GDH allostery and how it is correlated with biological function.

EXPERIMENTAL PROCEDURES

Mutagenesis

The antenna-less GDH construct was generated using overlapping PCR and the GDH1 containing pCMV-SPORT6 plasmid from Harvard PlasmidID (HsCD00338409). The product was then ligated into the pFastBac1 plasmid (Bac-to-Bac Vector Kit, Thermo Fisher Scientific, Inc). The primers used are shown in Table 1. For wt GLUD1, the assembled pFastBac donor plasmid was a kind gift from the Ioannis Zaganas and Andreas Plaitakis laboratories. All constructs were confirmed by sequencing the donor plasmids.

The generation of the R400Q antenna-less mutant was performed using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies). The template plasmid used was the aforementioned antenna-less GDH gene in the pfastBac1 plasmid.

The primers are shown in Table 1. Successful mutagenesis was confirmed by sequencing the donor plasmid.

Protein Expression and Purification

Proteins were expressed using the ExpiSf Baculovirus Expression System (Thermo Fisher Scientific) as previously described [101]. In brief, Sf9 cell derivatives transfected with baculovirus containing the antenna-less GDH gene were incubated for 3 days at which point there were clear signs of late stage infection. The cells were centrifuged (300g for 5 minutes) and supernatant harvested to generate the P0 viral stock. This stock was used to infect fresh Sf9 cells for protein expression. After a 3-day incubation period, the cells were centrifuged for 5 minutes at 300g and frozen at -20°C. For purification, the cell pellet was mixed with lysis buffer (50 mM potassium phosphate, pH 7.0, 0.5M sodium chloride, and protease inhibitor cocktail containing AEBSF, EDTA, Bestatin, Pepstatin A, and E-64) at a 20% w/v ratio of pelleted cells to buffer. The cells were subjected to three freeze-thaw cycles and sonicated. Using a thermocouple to monitor the processing, the temperature of the cells remained below 15°C throughout sonication. Debris was removed from sonicated cells by centrifugation at 7000g for 10 minutes at 4 °C. The GDH was precipitated from the supernate using a 60% ammonium sulfate saturated solution, final concentration. This greatly stabilized the protein and allowed indefinite storage at 4°. The precipitate was collected via centrifugation at 10,000g for 30 min at 4°C. The pellet was re-suspended in 100mM potassium phosphate, pH 7.0, and dialyzed overnight in the same buffer. Debris was removed from this dialysate via centrifugation, filtered, and purified by size exclusion chromatography using

a Superose-6 column (GE Healthcare Systems). Fractions containing activity were pooled and concentrated. While the Sf9 cells produce NAD(H) dependent GDH, indigenous enzymatic activity was not detectable in uninfected cells that had undergone mock purification.

Quantification of Protein Expression

Western blot analysis was used to determine relative protein expression levels of wt, antenna-less mutant, and the R400Q antenna-less mutant. Purified bovine GDH was used as a positive control and standard. Proteins were separated based on size using SDS-PAGE (15% acrylamide) and were electrophoretically transferred to a PVDF blotting membrane. After transfer, the membrane was blocked using 5% BSA in PBS overnight at 4 degrees. Blocking buffer was removed by extensive washing with PBS and 0.2% Tween 20. The primary antibody used was the Rabbit anti Glutamate Dehydrogenase 1 polyclonal antibody (Bio-Rad) at the recommended 1:1000 dilution and incubated for 2 hours at room temperature. The membrane was again washed extensively using the PBS and Tween buffer prior to the addition of the secondary antibody. The secondary antibody was the Goat Anti-Rabbit IgG H&L, HRP (Abcam) at a 1:30,000 dilution and was incubated for 2 hours at room temperature before it was extensively washed.

Detection of the HRP conjugate secondary antibody used a luminol based chemiluminescent substrate. The luminescent membrane was removed from substrate buffer and placed in a plastic sheet protector before it was exposed to X-ray film. Film was developed using the Protec EcoMax tabletop film processor.

Specific activities of the various forms of GDH were estimated by Western blot. Under the same conditions, enzymatic activity of the various samples of GDH were measure and then the same samples were analyzed by Western blot. The area of the bands were estimated using the program ImageJ [92], and the ratio of the activity:area was used to compare specific activities.

Steady State Analysis

For the kinetic analysis, rates were measured spectrophotometrically by monitoring NAD(P)H at 340nm using a Hitachi U-3010 spectrophotometer at room temperature. Unless otherwise noted, oxidative deamination reactions were performed in 0.1M potassium phosphate (pH 8.0) buffer containing 50mM glutamate and 0.5mM $NADP^+$. Reductive amination reactions were performed in a 0.1M potassium phosphate buffer (pH 7.0) buffer containing 0.1mM NADPH, 0.1M ammonium, and 5mM 2-oxoglutarate. All data was analyzed using the program Prism (GraphPad, Inc).

For the various analyses, the following equations were used;

Equation 1: Modified Hill equation – activation

$$\% \text{ Activation} = 100\% + \frac{A_{max}[A]^h}{ED_{50}{}^h + [A]^h}$$

In this equation, Amax is the maximum activation, [A] is the concentration of the activator, ED50 is the concentration that gives half maximum activation, and h is the Hill coefficient.

Equation 2: Modified Hill equation - inhibition

$$\%\ \text{Inhibition} = 100\% - \frac{I_{max}[I]^h}{{ED_{50}}^h + [I]^h}$$

Similar to equation (1), Imax is the maximum inhibition, [I] is the concentration of the inhibitor, ED50 is the concentration that gives half maximum inhibition, and h is the Hill coefficient.

Equation 3: Modified Hill equation

$$V = \frac{V_{max}[NADP^+]^h}{{K_m}^h + [NADP^+]^h}$$

This equation is the modified Hill version of the Michaelis-Menton equation and used to describe cooperative enzymes. Vmax is the maximum velocity, Km is the Michaelis-Menton constant for this reaction, [$NADP^+$] is the concentration of coenzyme, and h is the Hill coefficient.

Equation 4: Substrate Inhibition

$$V = \frac{V_{max}[Glu]}{K_m + [Glu](1 + \frac{[Glu]}{K_i})}$$

This equation is used to describe apparent substrate inhibition at high substrate (glutamate) concentrations. The variables are the same as equation (3) with the addition of Ki which is the apparent substrate inhibition constant.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

TABLES

Table 1: Primers to remove antenna

Step 1: Generate 2 fragments	
First Fragment	
Forward primer:	5'-ATGTACCGCTACCTGGGCGAAGCG-3'
Reverse primer:	5'-CAAACGGCCATAGCTGACATGATTTAGATTC-3'
Second Fragment	
Forward primer:	5'-GCATCTGAGAAAGACATCGTGC-3'
Reverse primer:	5'-TGTGAAGGTCACACCAGCTT-3'
Step 2: Add overlapping region	
First Fragment:	
Forward primer:	5'- ATGTACCGCTACCTGGGCGAAGCG-3'
Reverse primer:	5'-ACCCGATATCCTCAAACGGCCATAGCTGACATGATTTAGATTC-3'
Second Fragment	
Forward primer:	5'-TATGGCCGTTTGAGGATATCGGGTGCATCTGAGAAAGACATCGTGCACTCTG-3'
Reverse primer:	5'-GCTACTCGAGTCATGTGAAGGTCACACCAGCTT-3'
Step 3: Join two fragments and amplify	
Forward primer:	5'-TACTAGAATTCATGTACCGCTACCTGGGCGAAGC-3'
Reverse primer:	5'-ATGCTACTCGAGTTATGTGAAGGTCACACCAGCTTCATTGTACAC-3'

Primers to generate the R400Q antenna-less mutant

Forward primer:	5'-AATCATGTCAGCTATGGCCAGTTGAGGATATCGGGTGCAT -3'
Reverse primer:	5'-ATGCACCCGATATCCTCAACTGGCCATAGCTGACATGATT -3'

Table 2: Kinetic parameters for antenna-less investigation

	bGDH	Wt hGDH	Antenna-less GDH
ADP Reverse			
Kact	53±7.0μM	48±5.7μM	177±10μM
Amax	172±12%	277±16%	1,802±42%
Hill Coefficient, h	2.3±1.6	2.5±0.5	2.0±0.14
R^2	0.909	0.925	0.992
GTP Forward			
Kinh	31.1±3.9μM	15.3±0.38μM	16.6±2.3μM
Imax	100±4.0%	99.9±1.0%	100±4.0%
Hill Coefficient, h	1.07±0.10	2.06±0.08	1.4±0.13
R^2	0.980	1.000	0.9933
GTP Reverse			
Kinh	19.3±1.0μM	17.5±0.47μM	33±3.2μM
Imax	95.3±2.4%	95.9±1.2%	81.4±3.9%
Hill Coefficient, h	1.7±0.16	1.2±0.28	1.5±0.24
R^2	0.985	0.994	0.976
Glu inhibition			
Kinh	235±33mM	124±14mM	1,370±330mM
Km	1.8±0.23mM	1.7±0.21mM	8.5±0.9mM
R^2	0.929	0.954	0.985
Glu inhibition + ADP			
Kinh		818±126mM	241±25mM
Km		6.3±0.53mM	2.2±0.21mM
R^2		0.989	0.966
Negative Cooperativity			
Km	2.3±0.83μM	0.28±0.06μM	0.30±0.83μM
Hill Coefficient, h	0.67±0.07	0.68±0.06	0.52±0.06
R^2	0.997	0.997	0.987
Leucine Activation			
Kact	1.27±0.23mM	0.90±0.31mM	6.9±0.75mM
Vmax	152±6.4%	144±5.7%	513±13%
R^2	0.814	0.785	0.990
Bithionol Inhibition			
Kinh	1.2±0.17μM	1.6±0.04μM	1.3±0.04μM
Imax	99.7±7.1%	97.8±2.2%	82.3±2.2%
Hill Coefficient, h	1.2±0.39	1.6±1.6	5.0±0.76
R^2	0.992	0.987	0.987
EGCG Inhibition			
Kinh	2.0±0.06μM	3.7±0.23μM	3.1±0.52μM
Imax	97.0±1.1%	100±1.0%	89.3±5.6%
Hill Coefficient, h	2.5±0.16	1.5±0.11	0.92±0.15
R^2	0.9967	0.9846	0.9566

FIGURES

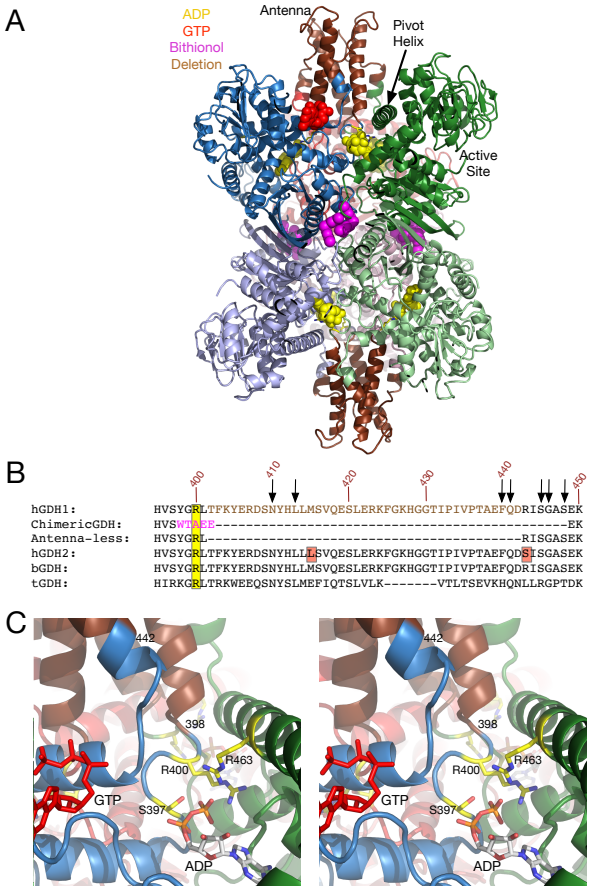

Figure 13. The structure of mammalian GDH and the region of interest in this study.

(A) Ribbon diagram of the GDH/ADP complex with each subunit colored in different hues. While this structure did not contain GTP or bithionol, they have been overlaid onto the ADP/GDH complex as points of reference. Bound ADP, GTP, and bithionol are represented in yellow, red, and mauve, respectively. The region deleted in this study is represented by a brown ribbon. (B) Sequence alignments of the antenna domain of the various forms of GDH using hGDH for numbering. The locations of HHS causing mutations are highlighted by black arrows and R400 is highlighted in yellow. The differences between isoforms hGDH1 and hGDH2 are highlighted in pink. (C)

Stereo image of the details of the ADP binding site. Key residues discussed in the text are also noted.

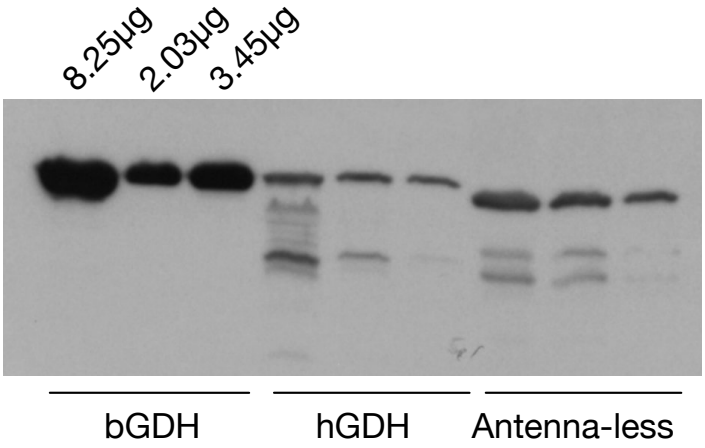

Figure 14**.** Western blot analysis of the various forms of GDH.

By quantifying the approximate amount of GDH in the various samples and comparing that to their respective enzymatic activities, the specific activity of wt hGDH expressed here was found to be equivalent to bGDH and the antenna-less form was only 13% as active as wt hGDH. For each sample, three different amounts were loaded onto the gel to ensure that at least some of the replicates were in the linear portion of the detection signal. Note that the molecular weight of the antenna-less form is smaller than wt, consistent with the deletion of the antenna. The smaller molecular fragments reacting in the Western blot represent proteolytic fragments not eliminated by the partial purification process.

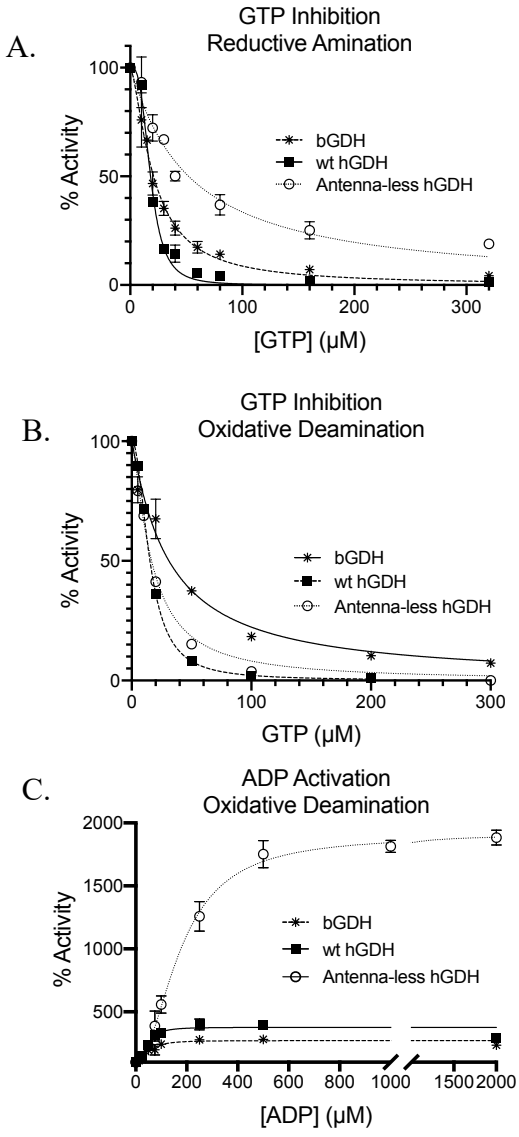

Figure 15. Effects of removing the antenna on GTP and ADP regulation.

(A) bGDH, wt hGDH, and the antenna-less mutant were all sensitive to GTP inhibition in the reductive amination reaction with the antenna-less mutant being slightly less sensitive. (B) In the oxidative deamination reaction, all three forms of GDH were essentially equal in GTP sensitivity. (C) In contrast to GTP, the ADP activates the antenna-less mutant 10-times more than either wt hGDH or bGDH.

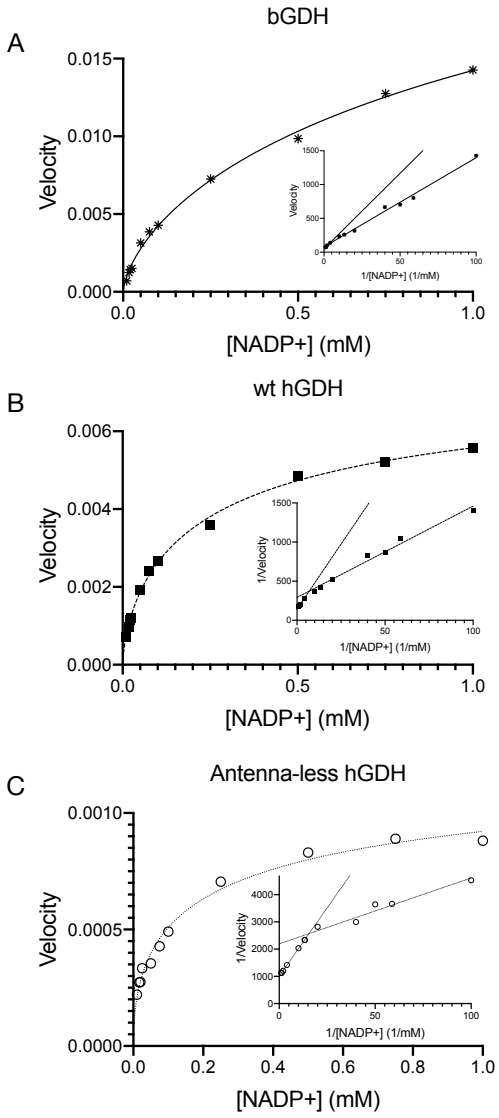

Figure 16. Effects of removing the antenna on negative cooperativity.

Shown here are the steady-state analyses of the oxidative deamination reaction with varied coenzyme concentrations using the bGDH, wt hGDH, and antenna-less hGDH in panels A, B, and C, respectively. The curves represent non-linear regression analysis using the modified Hill equation described in the Methods section. The insets show the same data plotted in Lineweaver-Burke format to exemplify the non-linearity

indicative of negative cooperativity. By all metrics, all three forms of GDH exhibit negative cooperativity.

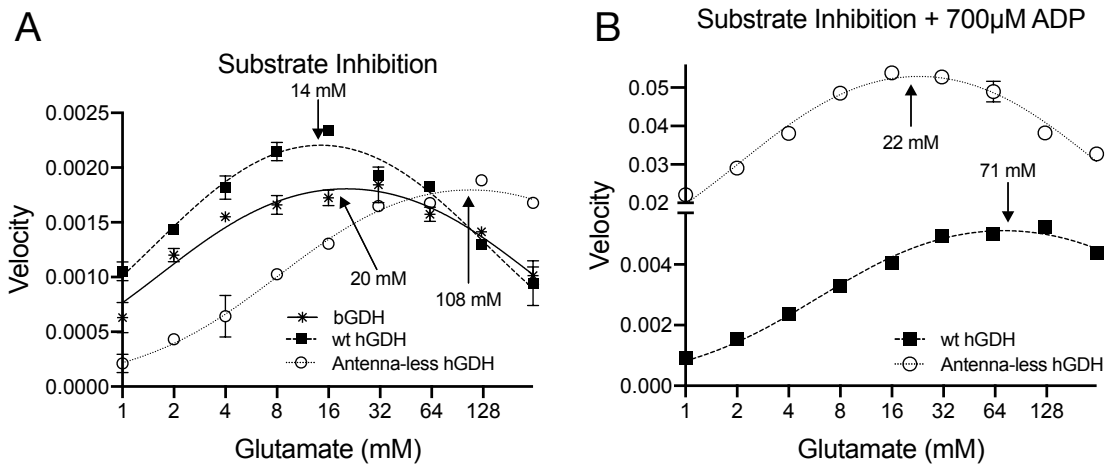

Figure 17. Effects of removing the antenna on substrate inhibition.

(A) All three forms of the enzyme exhibit typical substrate inhibition in the oxidative deamination reaction. However, as noted in the figures, it takes ~10 times more glutamate to see the same level of substrate inhibition as wt. (B) When ADP is added to the antenna-less GDH, the reaction is enhanced by more than 10-fold compared to wild type. As previously observed [15, 41], the addition ADP to wild type GDH increases the concentration required to observe substrate inhibition, indicative of an increase in the Km for glutamate.

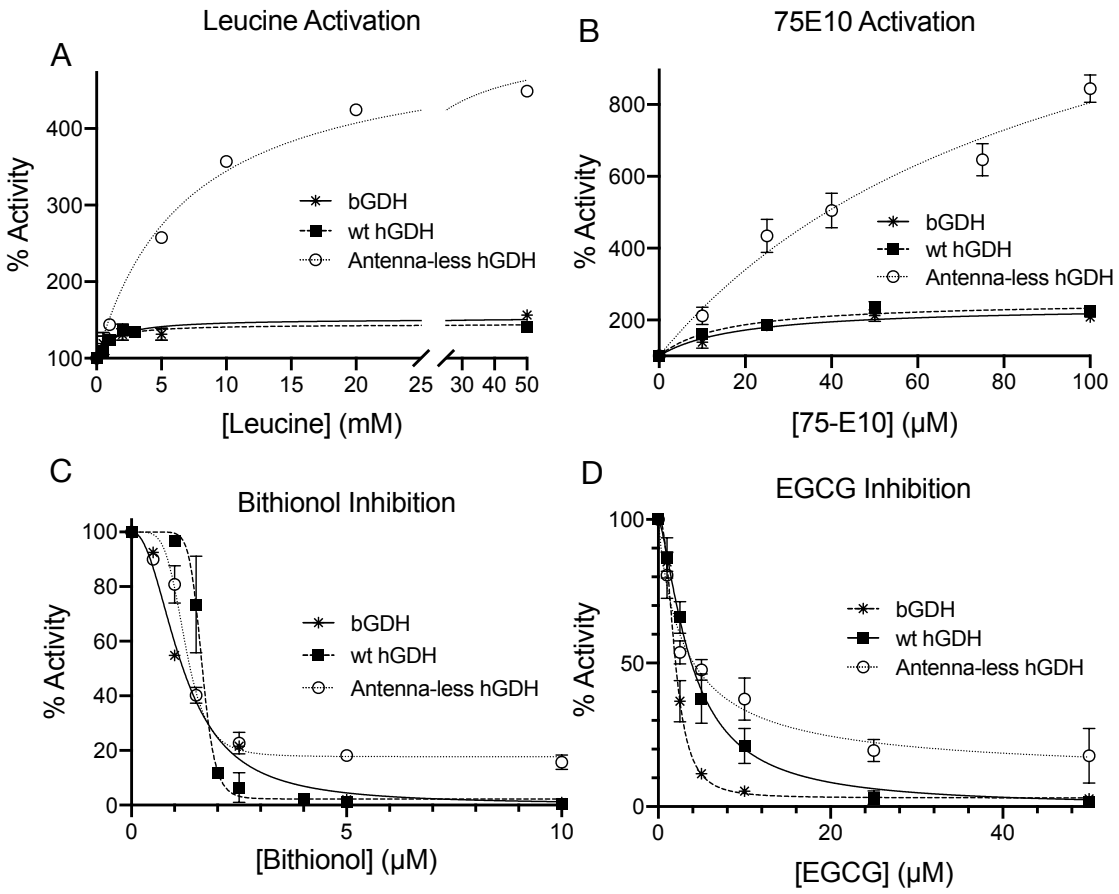

Figure 18. Effects of removing the antenna on other allosteric regulators.

(A) Leucine causes modest activation in wt GDH in the absence of inhibitors such as GTP. However, the antenna-less mutation is 10-fold more sensitive to leucine compared to wt. (B) Similar to leucine, the antenna-less mutant is more sensitive to the synthetic activator, 75-E10, than wt. (C) The antenna-less mutant is inhibited by biothionol (Figure 13) to approximately the same efficacy as wt. (D) EGCG inhibits by binding to the ADP site [37] and also inhibits the antenna-less form.

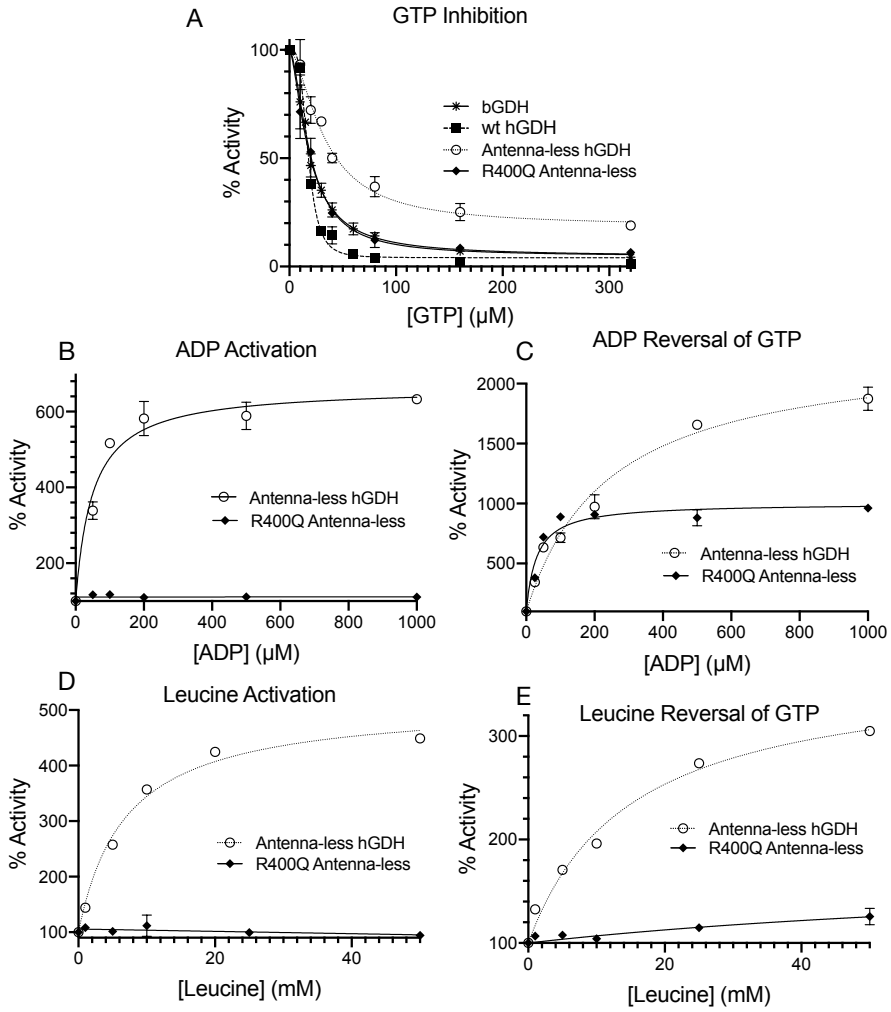

Figure 19: Effects of the R400Q mutation on allosteric regulation of antenna-less hGDH.

(A) GTP inhibits all four forms of GDH. (B) and (D) ADP and Leucine hyper-activate the antenna-less forms of GDH, but the R400Q eliminates both effects. (C) While the R400Q mutation blocks ADP activation, ADP is still able to abrogate GTP inhibition in the mutant. (D) The R400Q mutation blocks all forms of leucine activation including the abrogation of GTP inhibition.

Chapter 3: Mutations in Glutamate Dehydrogenase That Cause Hyperinsulinism-Hyperammonemia Elucidate the Role of the Antenna

Zoe A. Hoffpauir and Thomas J. Smith

ABSTRACT

Glutamate dehydrogenase is a homohexameric enzyme comprised of ~500 residue subunits and catalyzes the reversible oxidative deamination of glutamate. In animals, the enzyme exhibits complex allosteric regulation by a wide array of important metabolites[8]. The importance of this regulation was made evident by the hyperinsulinism/hyperammonemia syndrome (HI/HA) where mostly spontaneous mutations cause a gain of function in GDH [22, 59, 86]. The current model for this pathology is that higher catabolism of glutamate in the pancreas feeds more 2-oxoglutarate into the Krebs cycle leading to elevated ATP levels and secretion of insulin [45, 106]. A number of these HI/HA mutants are found in the antenna region, distal to all allosteric regulatory sites. The purpose of these studies is to examine the effects of several antenna HI/HA mutants to both understand the pathology of these variants as well as to shed light on the structural details of the role of the antenna in allosteric regulation. The mutants grouped into three main categories that all increased GDH activity; an increase in basal activity, an increase sensitivity to allosteric activators, or decrease in sensitivity to GTP inhibition. These mutants also demonstrate that single residue changes in the descending helix of the antenna can have profound effects on both catalysis and regulation. Finally, analyses of GDH inhibition by palmitoyl CoA (PCA) is unlikely to be true allostery since PCA irreversibly inactivates the enzyme. However, ADP and the P436L HI/HA mutant protects against this inactivation, suggesting some specificity in PCA binding.

INTRODUCTION

Glutamate dehydrogenase (GDH) catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate using $NAD(P)^+$ as a coenzyme. GDH is found in every living organism [8, 74], and in eukaryotes, GDH is located in the inner mitochondrial matrix where it feeds 2-oxoglutarate into the Krebs cycle linking amino acid catabolism to glycolysis and fatty acid catabolism [8, 107]. GDH from animals, but not other kingdoms [8, 10], is allosterically regulated by a variety of metabolites [2, 8, 10, 15, 46, 76]. These regulators are thought to work by modulating the rate-limiting step of GDH catalysis, product release, under most conditions [77]. Allosteric inhibitors GTP, and, with 100 fold lower affinity, ATP are thought to increase the binding affinity of the product to the active site, thereby slowing catalytic turnover [8, 10, 12]. ADP and leucine are allosteric activators of GDH and likely activate by facilitating product release [14, 15, 107]

The crystal structures of a number of different GDH complexes have been determined [5, 33-35, 37, 38, 102, 108, 109] (Figure 20). The enzyme is a homohexamer with each subunit being comprised of ~500 residues. The first ~200 amino acids form an N-terminal domain that is mainly comprised of long β-strands that form extensive inter-subunit interactions across the hexameric 2-fold axes. The next ~200 residues form the NAD binding domain that rotates ~18° during each catalytic turnover event. Rising up from the top of the NAD binding domain is the ~44-residue 'antenna' feature that is comprised of a long 'ascending helix' and returns toward the surface of the enzyme via a flexible coil at the outermost tip, leading into smaller 'descending helix'. The antenna domain appears to have evolved concomitantly with allosteric regulation [5]. The

descending helix is connected to the 'pivot helix' so named because it is convenient to consider the rotation of the NAD binding domain to be about this helix. Glutamate binds to the deepest recesses of the active site (Figure 20, yellow spheres) and, in the structure of this abortive complex (GDH/NADH/Glutamate) the coenzyme (black spheres) binds in an extended conformation. GTP (red spheres) binds at the base of the antenna between the pivot helix and the NAD binding domain. From structures of the apo [33, 34, 102] and GTP bound [35, 36] forms, it is clear that the GTP binding site is most available when the catalytic mouth is rotated down upon substrate and coenzyme. Shown in cyan is a second NADH/NAD binding site to which the activator, ADP, also binds. This allosteric site is a rather promiscuous site since the inhibitors EGCG and ECG also bind to this location [37].

The metabolic importance of the complex allosteric regulation found in animal GDH was made evident by the linkage between the hyperinsulinism/hyperammonemia syndrome (HI/HA) and gain of function mutations in GDH. Upon consumption of protein, HI/HA patients present insulinemia and hyperammonemia followed by hypoglycemia [21]. This is likely due to GDH hyperactivity in the β-cells causing increases in glutamate catabolism, feeding more 2-oxoglutarate into the TCA cycle, increasing the ATP/ADP ratio, resulting in the closing of the ATP gated potassium channels. This depolarization of the β-cell membrane opens the voltage gated calcium channels, resulting in the release of insulin [21]. In the liver and kidneys, hyperactive GDH generates ammonia from glutamate catabolism while disrupting ureagenesis by depletion of N-acetylglutamate that, together, results in hyperammonemia [22, 110]. Because of the critical role that glutamate plays in the central nervous system, patients

also have increased risk of cognitive developmental defects and spontaneous seizures that are independent of hypoglycemia [54, 111].

Genetic analysis shows that there are at least 16 different residues on GDH that, when mutated, result in HI/HA [40, 59, 60]. These gain of function mutations in GDH are mostly spontaneous with a small number of hereditary cases observed in the less severe variants [58]. The hyperactivity of GDH in HI/HA is thought to be caused by a loss or decrease in sensitivity to GTP inhibition. With at least six of the HI/HA mutation sites, this is likely due to direct disruptions in the GTP binding site as was the case with the well-studied H454Y mutation [39]. However, many HI/HA lesions lie in the antenna and do not contact any known allosteric binding sites [5, 40]. We have shown that the antenna plays a major role in allosteric regulation likely via affecting the energetics of the extensive conformational changes required for catalysis [5, 40]. In the work presented here, analyses of these antenna HI/HA mutants not only lend insight into how these lesions affect GDH regulation, but also offer insight into the fine details of the role of the antenna in GDH catalysis and regulation. Finally, the inhibition of GDH by palmitoyl CoA (PCA) was reexamined. The irreversible nature of PCA inhibition was confirmed and protection by ADP and the HI/HA P436L mutant suggests a possible role of the antenna in PCA inactivation.

MATERIALS AND METHODS

Mutagenesis

Each of the HI/HA mutant DNA constructs was generated using the Agilent QuikChange II site directed mutagenesis system (Agilent Technologies). The template for all mutagenesis was the assembled pFastBac1 plasmid containing the GLUD1 gene with the mitochondrial import leader sequence [101, 112]. The protocol supplied by the company was followed with the addition of 3% (per volume) DMSO to the PCR reaction mix. The sequences of all mutated clones were confirmed by Sanger sequencing. The primers used for mutagenesis are shown in Table 3.

Protein Expression and Purification

Wild type human GDH (wtGDH) and HI/HA mutants were expressed using the ExpiSF Baculovirus expression system (Thermo Fisher Scientific) as previously described [40]. In brief, wtGDH or mutated GDH genes were moved from the shuttle vector to the bacmid via homologous recombination as per manufacturer instructions. SF9 cell derivatives were then transfected with these bacmids and incubated for 3 days. When signs of late stage baculovirus infection was visually evident, the cellular debris from transfected cells was removed via centrifugation and the supernatant was kept as the P0 viral stock for subsequent infections.

Frozen cell pellets of infected cells were thawed in 50mM potassium phosphate buffer, pH 7.0, containing 500mM sodium chloride and Pierce (Thermo Fisher Scientific)

protease inhibitor cocktail and subjected to three freeze/thaw cycles. The sample was then sonicated extensively using the Qsonica Sonicator and cellular debris was removed by centrifugation at 12,000xg for 10 minutes. GDH was precipitated from the supernatant with 60% ammonium sulfate, final concentration. GDH is stable in this ammonium sulfate slurry and was stored at 4°C. Prior to use, precipitated GDH was collected by centrifugation at 21,000xg for 10 minutes at 4°C and the supernatant removed. The pellet was resuspended in 100mM potassium phosphate, pH 7.0, and debris that did not resuspend was removed by centrifugation.

Western Quantification

Western blot analysis was used to determine the specific activities of the various HI/HA mutants compared to wtGDH as previously described [40]. In brief, samples were run on SDS-PAGE (15% acrylamide) and transferred onto polyvinylidene fluoride membranes. The membrane was blocked overnight in 5% powdered milk dissolved in PBS at 4°C and the membranes were extensively washed with PBS containing 0.2% Tween 20. The primary antibody, rabbit anti-glutamate dehydrogenase I polyclonal antibody (BioRad), was added at the recommended 1:1000 dilution and incubated for 2 hours at room temperature and washed with the PBS tween buffer. The presence of rabbit antibody was detected using goat anti-rabbit IgG (heavy and light chains), horseradish peroxidase (HRP)-conjugated antibody (Abcam, Inc.) at a 1:30,000 dilution using the luminol based chemiluminescence and X-ray film. The densities of the Western blot bands were quantified using ImageJ [92] and compared to initial rate velocities for specific activity estimates.

Steady State Analyses

Steady state velocities were measured spectrophotometrically as previously described [40]. Assays were performed in duplicate by monitoring NAD(P)(H) oxidation/reduction at 340 nm using a Hitachi model U-3010 spectrophotometer at room temperature. All data were analyzed using Prism (GraphPad, Inc.). For analysis of the steady state velocity with varying inhibitor concentrations, the following modified Hill equation was used for non-linear least squares fitting;

Eq #1: $$\%Activity = 100\% - \frac{Imax*[inhibitor]^h}{{K_I}^h+[inhibitor]^h}$$

For analysis of the enzymatic activity with varying concentrations of activator;

Eq #2: $$\%Activity = 100\% + \frac{Vmax*[activator]^h}{{K_{act}}^h+[activator]^h}$$

RESULTS

BASAL ACTIVITIES OF VARIOUS HI/HA MUTANTS

Before analysis of the allosteric regulation of the various HI/HA mutants, it was first necessary to measure their basal activity. Since contaminants in each sample could affect estimations of GDH content, a quantitative Western analysis was performed (Figure 21). Varying amounts of each GDH sample was separated via SDS-PAGE and the amount of GDH was estimated using the program ImageJ [92]. The activity of these samples was divided by estimated quantity of GDH and compared to expressed wild type huGDH. (Table 4). S445L and G446R have specific activities comparable to wtGDH. In contrast, P436L and S448P have only 20% and 50% the activity of wtGDH, respectively. The relative activity of S448P is in agreement with previously published work demonstrating S448P had significantly lower basal activity than wtGDH [39]. Interestingly, F440L has twice the specific activity of wtGDH. HI/HA is believed to be caused by a gain of function in GDH and, as will be shown in subsequent results, this doubling of specific activity in F440L appears to be the major difference to wild type. Therefore, while the other mutations may cause HI/HA by disrupting allosteric regulation, F440L may be directly increasing GDH activity.

ADP activation

The effects of the HI/HA mutations cause varied effects on ADP activation (Figure 22, Table 4). Note that the curves for S448P and P436L were separated from the other data sets because of the marked differences in scale. The F440L, G446R, and the S445L mutants were all activated to approximately the same extent as wild type and with approximately the same apparent affinity. The S448P mutant was activated by more than

six times that of wild type with only about two-fold decrease in apparently binding affinity. The P436L mutant was even more sensitive to ADP with more than a 36-fold increase in the extent of ADP activation with a decrease in apparently affinity for ADP by approximately 5.5-fold. The loss in basal activity and marked increase in ADP activation of the S448P and P436L mutants is very similar to the antenna-less form of GDH. It is unclear why the increase in extent of activation by ADP is also associated with apparently weaker binding. Nevertheless, like the antenna-less version of huGDH, these lesions create a slower form of the enzyme that is more sensitive to ADP activation.

Leucine Activation

Leucine is a poor substrate for GDH and also an allosteric activator of the enzyme [2]. In Thermus thermophiles, a thermophilic bacterium, GDH is activated by leucine akin to animal GDH and binds leucine at the interfaces of the subunits within a monomer near the binding site of previously identified inhibitors bithionol and GW5074 [38, 99]. The leucine binding site in human GDH is currently unknown, but it is believed to be distinct from the ADP binding site [17].

The trends with of the effects of the HI/HA mutants are similar to ADP activation. Most of the mutants did not show a significant difference in their sensitivities to leucine activation (Figure 23). Interestingly, the G446R mutant appears to have ~5-fold weaker affinity with both ADP and leucine, even though they are unlikely to bind to the same site. Also, similar to ADP activation, both the P436L and S448P mutants are hypersensitive to leucine. Indeed, the P436L mutation is even more sensitive to leucine activation than our previous antenna-less form of GDH [40].

GTP inhibition

To a first approximation, the effects of the mutations on GTP inhibition were opposite to their effects on ADP and leucine activation (Figure 24, Table 4). While the F440L, S445L, and G446R mutations had the smallest effects on ADP/leucine activation, they had the largest effects on GTP inhibition with increases in apparent affinity (Ki) for GTP by 5, 10, and 20-fold, respectively. The S448P mutation slightly disagreed with this generalization with a modest increase of the Ki for GTP of ~3 fold and a 23% loss in maximum GTP inhibition. With all mutations, the effects on the extent of GTP inhibition were less pronounced than the apparent affinity and losses in maximum inhibition varied from 5 to 24%.

Palmitoyl CoA (PCA) Inhibition

As shown in figure 25 and table 4, PCA inhibition curves markedly deviate from simple asymptotic curves approaching zero. Therefore, the modified Hill equation (equation #1) was used for fitting and showed that all GDH samples exhibited apparent positive heterotrophic cooperativity. The only mutation that had significant effects on PCA inhibition was P436L with more than a 4-fold increase in Ki.

Unlike GTP inhibition, previous studies suggest that PCA effects on GDH may not be truly allosteric. Centrifugation studies demonstrated that PCA breaks the hexamer apart and irreversibly inactivates the enzyme [12]. This is not a universal effect of PCA on dehydrogenases since inhibition of malate dehydrogenase was fully reversible.

Subsequently, it was shown that allosteric regulators such as ATP, GTP, and leucine decrease PCA inhibition and that ADP might be directly competitive with PCA [78]. However, the possible binding site of PCA on GDH is unknown.

In light of the P436L effects on PCA inhibition, the details of PCA inhibition was reexamined. Shown in figure 26 is the effect of ADP on GDH activity in the presence and absence of PCA. As expected under these conditions, ADP activated the enzyme by ~2-fold. If GDH was pre-incubated with PCA and then ADP was added in the assay, the enzyme was essentially inactive and ADP did not abrogate PCA inhibition. However, if the same amount of ADP was added to the enzyme first and then PCA was added, ADP appeared to activate the reaction in a dose-dependent manner. Since the order of addition is critical, it is more than likely that PCA denatures the enzyme and ADP offers some protection from this inactivation process. Therefore, PCA inhibition of GDH needs to be considered with caution since it appears to not be a truly reversible allosteric inhibitor. This denaturation process could explain the marked positive cooperativity in that the enzyme may require a certain level of saturation with PCA before the denaturation process begins.

DISCUSSION

What is clear from these studies is that this group of HI/HA mutants all cause some gain of function in GDH, but do so by quite different mechanisms. Since we have shown that GDH is a highly dynamic enzyme and this descending helix undergoes significant conformational changes during each catalytic cycle [8], the locations of the mutations must be considered in both the ‘open’ and ‘closed’ catalytic mouth

conformations. Figure 27 shows the structure of GDH around the descending helix in the open (darker hues) and closed (lighter hues) conformations. Figure 27A shows the locations of the HI/HA mutants and the grey arrows denote the conformational changes in the loop connecting the descending and pivot helices as the catalytic mouth closes. Figure 27B shows modeled HI/HA mutations as they might appear in the closed conformation. For this figure, the closed conformation was chosen since the G446R and S445L mutations undergo the most drastic environmental changes, going from solvent exposed to being buried in the antenna as the cleft closes. The side chains were simply placed in one of their preferred rotameric positions without further energy refinement. The effects of each mutant on GDH activity will be discussed in the context of these atomic structures.

S448 lies at the C-terminal end of the loop between the descending helix and the pivot helix. As noted in Figure 27, this loop is highly mobile as the catalytic mouth opens and closes. The S448P mutation causes at least a 50% loss in basal activity and slight abrogation of palmitoyl-CoA and GTP inhibition. What is notable is that the apparent efficacy of the activators ADP and leucine is markedly increased by 4-5 fold. The mutation to proline most certainly makes this connecting loop less flexible and since movement in this loop is essential for the open/close conformational changes during the catalytic cycle, it is not surprising that the S448P would decrease basal activity. Indeed, this loop is more extended in the closed conformation as the pivot helix shifts away from the antenna. In the open conformation, this loop is more contracted as the descending helix forms an additional turn. Indeed, if the S448P is modeled and simple regularization is performed in both conformations, the resulting proline tends to adopt the less favorable

cis conformation in the closed conformation and trans in the open. In total, the effects of this mutation may be akin to our previous studies where most of the antenna was removed [5, 40]. Without the antenna, it was argued, the enzymatic efficiency is diminished because of difficulty in moving the NAD binding domain. This could be much the same if the S448P mutation in this loop makes the structural transition more difficult. As with the antenna deletion, ADP may compensate for this by decreasing the energy required for NAD binding domain movement.

The S445L and the G446R mutations are also in this same connecting loop, but their effects are significantly different. With these two mutants, the Ki for GTP increases by nearly ten-fold but there were modest or no effects on ADP, leucine, or Palmitoyl CoA regulation. As with the S448P mutation, the effects of these mutations may be on the conformational transitions during catalysis since the environments of these residues are strikingly different in the open and closed conformations. In the open conformation, both S445 and G446 are on an additional helical turn and both are exposed to solvent at the base of the antenna. However, when the mouth closes, these residues rotate back towards the antenna interior. S445 moves into a hydrophilic environment including R407. Therefore, an S445L mutation would place the hydrophobic leucine into a crowded and charged environment. G446 moves into a pocket containing a cluster of acidic residues that we previously showed to bind europium [109] that alleviates zinc inhibition of GDH. Therefore, the G446R mutation might make closure of the catalytic mouth unfavorable because it places a large side chain into a crowded environment. Alternatively, it might even be possible that the arginine mimics europium. Eu^{3+} binds to the three E406

residues inside the base of the antenna trimer and abrogates Zn^{2+} inhibition that binds to the GTP site (Figure 27B, [109]).

The effects of the F440L mutation on GDH regulation appear to be limited to an increase in the Ki for GTP by approximately 5-fold. Compared to the carboxyl end of the descending helix, the N-terminal portion moves slightly towards the ascending helix as the catalytic mouth opens and closes. In addition, the F440L mutation is a rather conserved change and causes an increase in the Ki for GTP. As with the other mutations that affect the Ki for GTP, it is not clear whether the F440L mutation effects are on GTP binding or the subsequent allostery that results in inhibition. From structural and biochemical analysis [8, 33, 35, 36], the closed conformation has a more open binding site for GTP binding. Therefore, these mutations, distal to the GTP binding site, could affect the opening of the GTP binding site. Interestingly, this mutant is approximately twice as active as the wild type. It may be that replacing that large phenylalanine at the top of the descending helix with a smaller leucine allows for more facile conformational changes during catalysis. Therefore, this mutant has a double HI/HA effect by increasing the basal activity and making the enzyme less sensitive to GTP.

The P436L mutation has a profound effect on all types of allosteric regulation except GTP inhibition. P436 is at the top, N-terminal end, of the descending helix. The proline forms a sharp turn that positions the descending helix at an angle to the ascending helix and the side chain points down towards several hydrophobic residues at the interior of the antenna. The P436L mutant is clearly deleterious since it only has 20% the basal activity of wild type with unchanged Imax and Ki for GTP. However, this form of enzyme is hypersensitive to both leucine and ADP activation and far less sensitive to

PCA inhibition. Since PCA may be causing irreversible denaturation of GDH, it is interesting that this P436L mutant is far less sensitive to PCA.

PCA Inhibition

From these and previous studies [12], PCA effects should probably not be considered allosteric. The earlier studies showed that at a stoichiometry of 1PCA:1GDH subunit, the enzyme irreversibly disassociates into dimers. Interestingly, they also found that it may not be a simple detergent effect since sodium dodecyl sulfate breaks the hexamer into individual subunits. It is unlikely that PCA exhibits true positive cooperativity in the kinetic studies. It is more probable that the very low concentrations of PCA do not inhibit the enzyme but once the hexamer starts to disassemble at higher concentrations, the activity quickly declines in an apparently cooperative manner. Nevertheless, ADP and the P436L mutant offer significant protection against this denaturation even though these two sites are quite distal to each other. It is tempting to speculate that the PCA binding site(s) might be in the antenna region. PCA might disrupt the antenna interactions that hold the trimers together while not affecting the extensive β-strand interactions at the dimer interfaces. As we have shown structurally, ADP binding to GDH favors the open conformation [34] and it may be the antenna is less accessible to PCA binding in that conformation. The larger physiological question is what, if any, role PCA plays in GDH regulation in-vivo? It seems unlikely that animals evolved a process of GDH regulation that involves irreversible denaturation. However, it is important to note that the inner matrix of the mitochondria has such a high concentration of protein it is a gel-like environment rather than the low protein conditions used in-vitro. Therefore,

it is not at all clear if GDH in the matrix is disassembled by PCA. Further, the concentration of free PCA in the mitochondria is unclear as are the effects of possible GDH complexes with other mitochondrial enzymes. Nevertheless, future in-vitro analyses that assume PCA is an allosteric regulator should be regarded with caution.

SUMMARY

Generally, these mutants fall into two groups. P436L and S448P retain their sensitivity to GTP inhibition while being hypersensitive to ADP and leucine activation. In contrast, F440L, S445L, and G446R lose sensitivity to GTP by virtue of higher Ki's but retain their activation by leucine and ADP. F440L has an additional activatory effect with a basal rate twice that of wild type. All told, these results clearly show that HI/HA lesions in GDH result in gain of function, but by quite different mechanisms. Therefore, future drugs to control HI/HA will need to inhibit the enzyme independent of all of these physiological allosteric regulator sites.

What is more difficult to generalize is how these mutations, distal to the GTP and ADP sites, can cause such profound effects on allosteric regulation. The descending helix undergoes large conformational changes during each enzymatic cycle as it extends when the catalytic mouth closes and recoils as the mouth opens. As with the antenna-less mutants [5, 40], these results clearly show that the antenna, particularly the descending helix, plays a crucial role in these conformational changes and, in turn, both catalysis and allosteric regulation. Since we have only two static structures to understand a complex conformational transition, fuller understanding of how these mutations and the antenna

affect catalysis and allostery will require extensive dynamic simulations and energy calculations (e.g. [102, 113]).

FIGURES

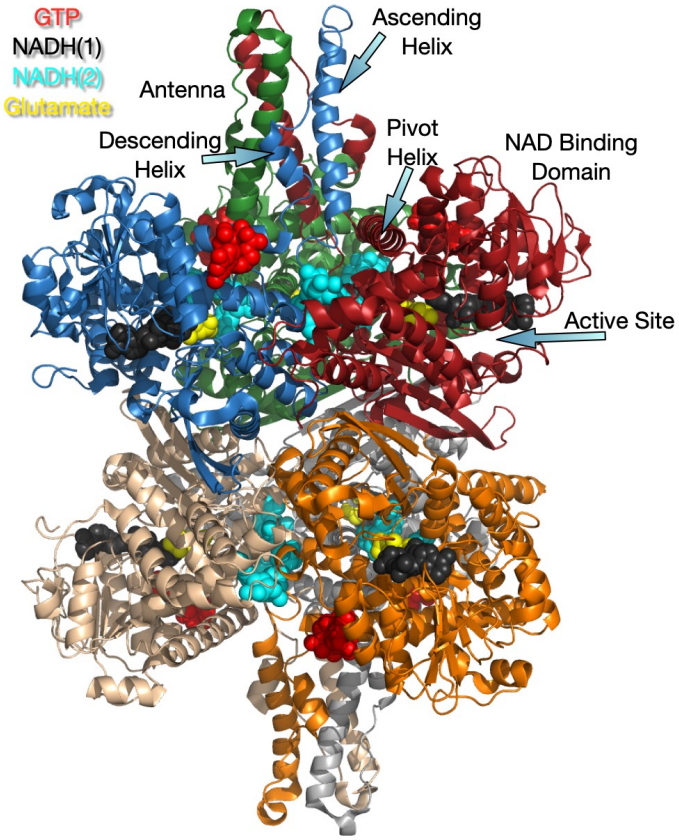

Figure 20: Ribbon diagram of the homohexameric structure of GDH

Shown here is a ribbon diagram of the homohexameric structure of glutamate dehydrogenase [PDB 6DHD [35, 36, 102]]. Each subunit is represented by individual colors and the locations of active site NADH, active site glutamate, the allosteric inhibitor GTP, and the second bound NADH in its inhibitory site are represented by black, yellow, red, and cyan spheres, respectively. Additional structural features are labeled accordingly.

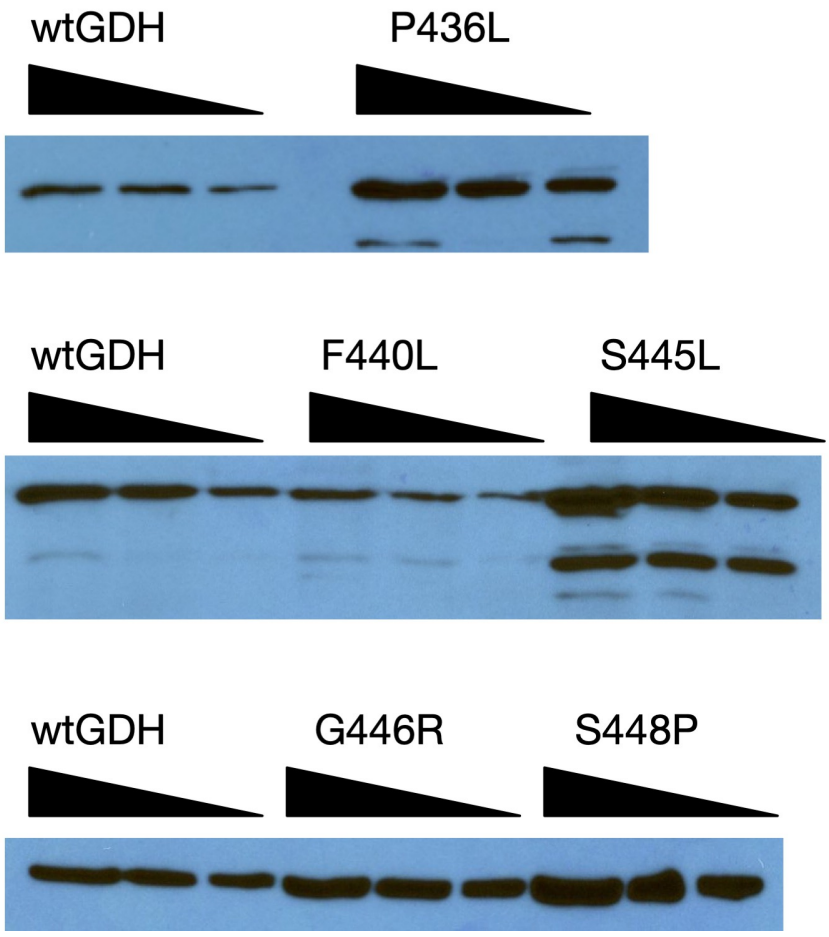

Figure 21: Quantitative Western blot analysis of the various forms of huGDH.

For each baculovirus expressed form of huGDH, varying amounts of protein were analyzed via SDS-PAGE and Western blotting. The bands corresponding to the full-length forms of GDH were quantified using ImageJ for specific activity estimations. Notably, the S445L mutant appears to be more sensitive to proteolysis during purification than the other forms but only the larger molecular weight band was used for quantitation.

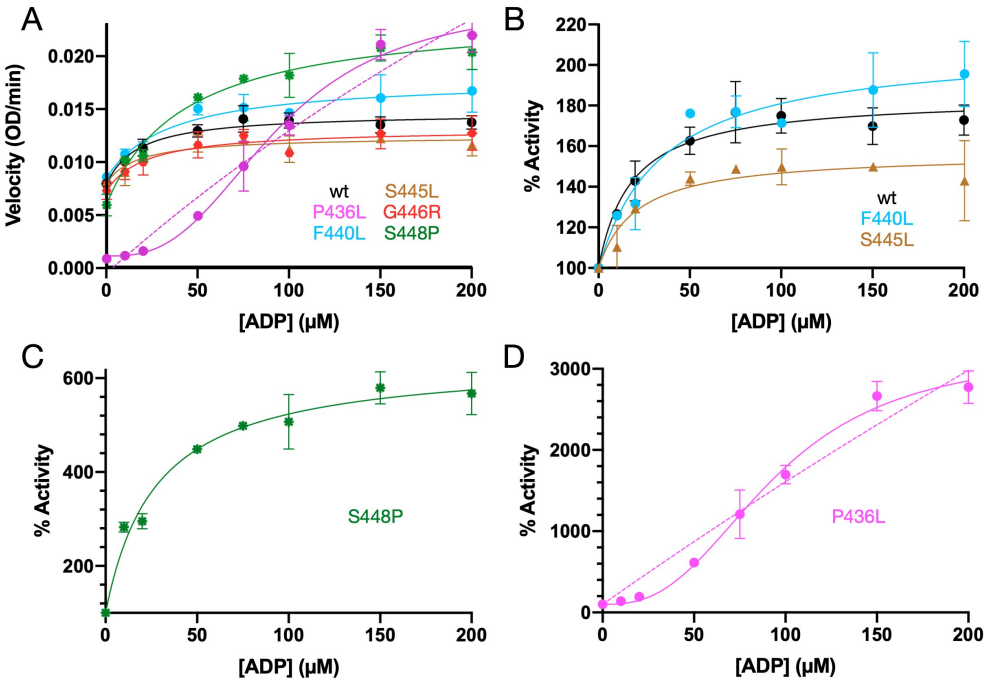

Figure 22: ADP activation of the HI/HA mutants.

A) Steady state velocities of the various forms of GDH at varying concentrations of ADP. Panels B-D show the same data plotted as percent activity to exemplify the extent of ADP activation. Since the percent activation differed so greatly, the data is presented on three different graphs. In panel D, the data was fitted to a simple activation curve (dashed line, equation 2, Hill coefficient =1) or the modified Hill form (solid line, equation 2).

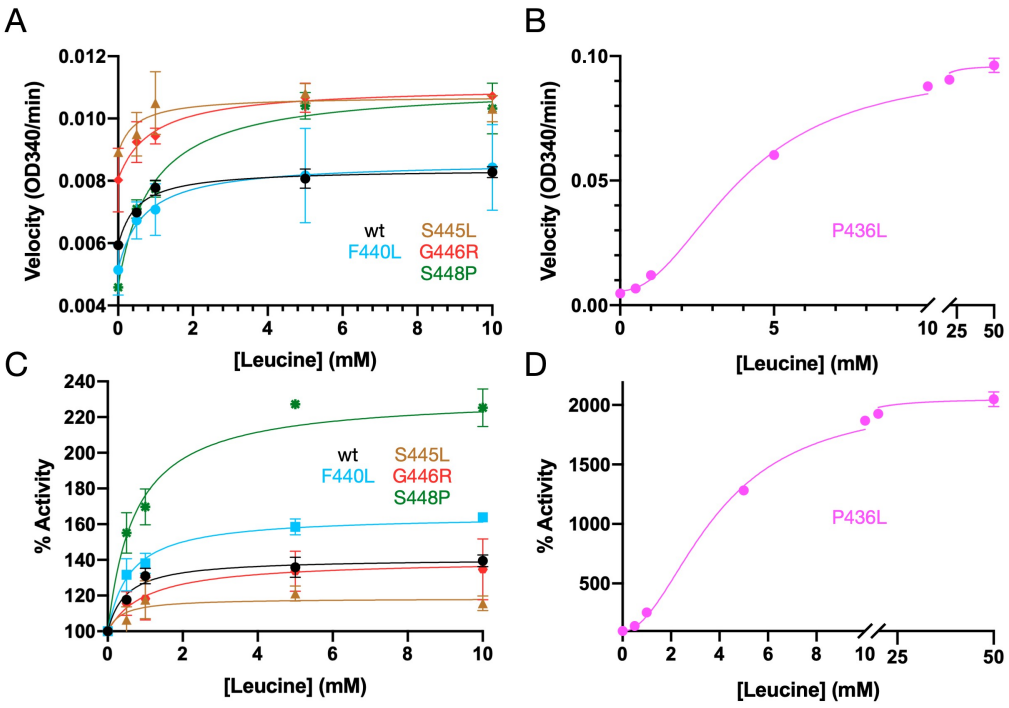

Figure 23: Leucine activation of the HI/HA mutants.

Panels A and B show the steady state velocities of the various forms of GDH in the presences of increasing concentrations of leucine. Panels C and D show the same data presented as a percent of the activity in the absence of leucine. The data for P436L was separated for clarity since leucine activated the enzyme to such a high degree.

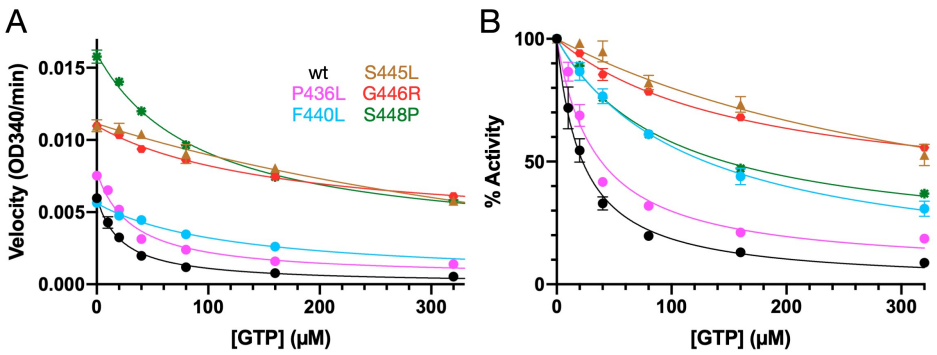

Figure 24: Effects of the various HI/HA mutants on GTP inhibition.

Figure A shows the raw velocities of the HI/HA mutants at varying concentrations of GTP. Figure B shows the same data plotted as percent activity in the absence of GTP. The estimated Ki and maximum inhibition for GTP from this data is summarized in Table 1.

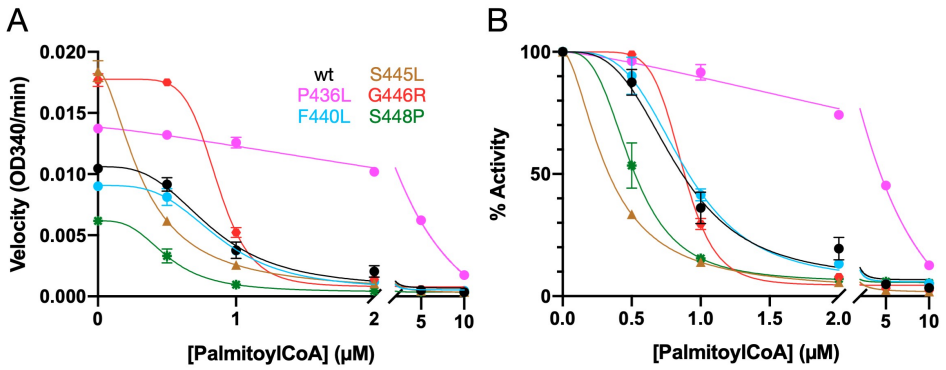

Figure 25: Sensitivity of the HI/HA mutants to palmitoyl CoA inhibition.

From the shape of the curves, palmitoyl CoA inhibition appears to exhibit positive cooperativity. This could be due to inter-subunit communication or effects other than allosteric inhibition such as denaturation. As shown in these graphs, the F440L mutant was nearly identical to wild type GDH, S448P and S445L were slightly more sensitive, while the P436L mutant was far more resistant to palmitoyl CoA inhibition (Table 1).

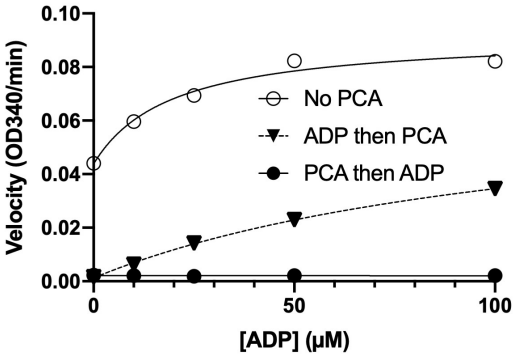

Figure 26: Palmitoyl CoA (PCA) inhibition of huGDH and reversal of that inhibition by ADP.

Panel A shows the steady state velocity at varying ADP concentrations in the presence and absence of 1µM PCA. As expected in these conditions, ADP activates by about 2-fold in the absence of any other additive. However, if PCA is added to the enzyme first, and then ADP is subsequently added in the assay, there is no evidence of activation or reversal of PCA inhibition. In contrast, if ADP is added to the enzyme, incubated and then PCA is added, the enzyme is at least partially protected. This suggests that PCA inhibition is not entirely allosteric or reversible.

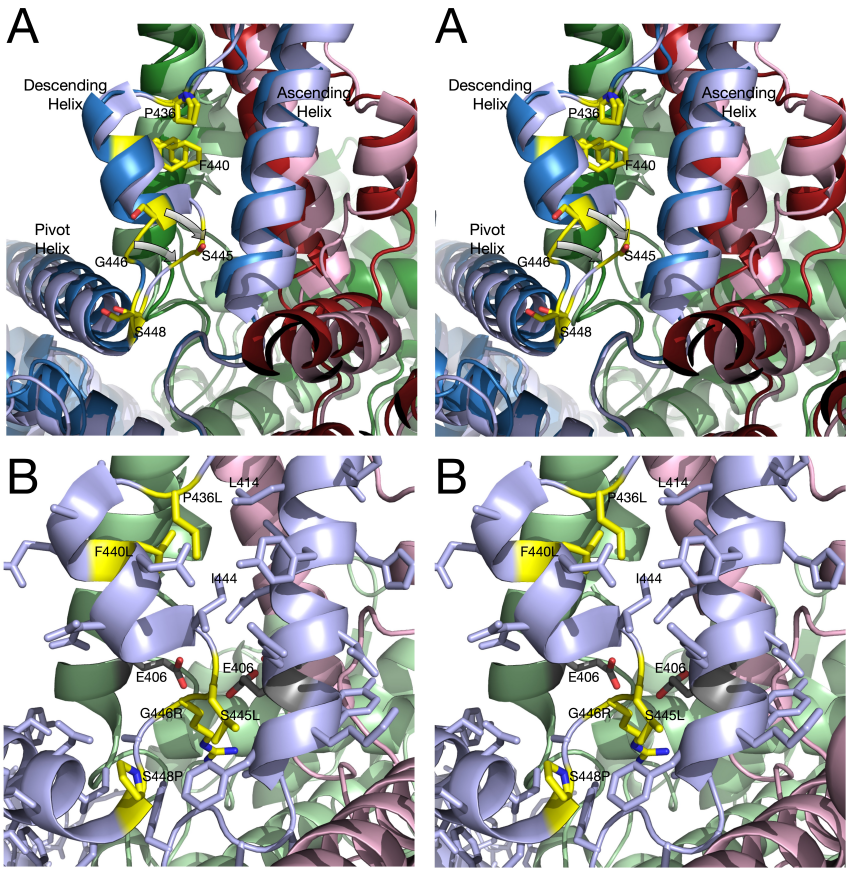

Figure 27: Shown here are stereo diagrams of antenna region where the HI/HA mutants are located.

A) Shown here is the superimposition of the open (darker hues) and the closed (lighter hues) conformations. The grey arrows denoted the large motion in the loop connecting the descending and pivot helices as the catalytic mouth closes. B) This stereo figure shows a model of the HI/HA mutations and their local environment in the closed conformation.

TABLES

Table 3: Primers used to generate the HI/HA mutants.

Mutagenesis Primers	
P436L Reverse	5'-CTTGAACTCTGCCGTGAGTACAATGGGAATAGTT-3'
P436L Forward	5'-AACTATTCCCATTGTACTCACGGCAGAGTTCCAAG-3'
F440L Reverse	5'-GATATCCTGTCTTGGAGCTCTGCCGTGGGTACA-3'
F440L Forward	5'-TGTACCCACGGCAGAGCTCCAAGACAGGATATC-3'
S445L Reverse	5'-TCAGATGCACCCAATATCCTGTCTTGGAACTCTGCC-3'
S445L Forward	5'-GGCAGAGTTCCAAGACAGGATATTGGGTGCATCTGA-3'
G446R Reverse	5'-GTCTTTCTCAGATGCACGCGATATCCTGTCTTGGA-3'
G446R Forward	5'-TCCAAGACAGGATATCGCGTGCATCTGAGAAAGAC-3'
S448P Reverse	5'-CACGATGTCTTTCTCAGGTGCACCCGATATCCTGT-3'
S448P Forward	5'-ACAGGATATCGGGTGCACCTGAGAAAGACATCGTG-3'

Table 4: Kinetic parameters for the HI/HA mutants of the descending helix

	wtGDH	P436L	F440L	S445L	G446R	S448P
Basal Activity (%)	100	20	200	100	100	50
ADP Activation						
Vmax(% Activation)	85±7.5	3,114±200	110±11	59±7.8	81±9.0	534±32
h	=1	2.7±0.4	=1	=1	=1	=1
Kact (μM)	17±4.0	94±6.0	34.0±8.7	19±5.6	22±6.2	29±5.6
Leucine Activation						
Vmax(% Activation)	39±1.3	1952±34	65±3.6	18±8.9	59±14.5	129±5.4
h	2.1±0.6	2.0±0.2	1.0±0.3	9.7±4.9	0.4±0.2	1.2±0.3
Kact (mM)	0.5±0.1	3.9±0.2	0.6±0.2	0.5±0.2	4.9±2.4	0.7±0.1
GTP Inhibition						
Imax (% Inhibition)	94±2.6	82±1.9	88±5.9	89±19	70±8.6	71±1.7
h	1.3±0.2	1.7±0.2	1.1±0.1	1.3±0.2	1.0±0.1	1.3±0.1
KI (μM)	20±1.5	26±1.4	99±13.9	291±85	185±42	70±3.3
Palmitoyl CoA Inhibition						
Imax (% Inhibition)	=100	=100	=100	=100	=100	=100
h	2.5±0.4	1.7±0.1	3.0±0.4	1.5±0.1	6.5±1.0	2.4±0.4
KI (nM)	900±56	4,009±180	931±40	318±15	878±29	523±38

Chapter 4: Conclusion and Perspectives

The complex allosteric regulation of animal glutamate dehydrogenase has been studied for decades yet remains enigmatic. One of the most perplexing aspects of GDH allostery is understanding how and why extensive allosteric regulation evolved. By examining a structural feature that is unique to allosterically regulated forms of the enzyme, the antenna domain, this current investigation refines previous understanding of the evolution of GDH allostery and seeks to elucidate the role of the antenna in allosteric regulation as a whole and the roles of the individual residues of the antenna in regulation and disease. The findings demonstrate that the antenna domain does not facilitate allosteric regulation. Instead, it tunes the basal catalytic activity of the protein and the extent of regulation by allosteric regulators.

To better understand the role of the antenna in allosteric regulation, it was removed from the protein and characterized. A previous study removed the antenna at its base and added 5 residues found in bacteria to bridge the gap left between two alpha helices of the NAD^+ binding domain as a result of antenna removal [5]. The resulting protein of the previous investigation did not exhibit allosteric regulation except by the activator leucine. The conclusion from this investigation was that the antenna evolved to facilitate allosteric regulation by GTP, ADP, and palmitoyl-CoA but that leucine activation was independent of the antenna and was likely layered on later in the evolutionary development of GDH. In the current investigation, the antenna was removed such that the residues at the base of the antenna were left intact to bridge the gap previously filled by the bacterial sequence. The biochemical properties of the new antenna-less construct are unexpected and exciting. Unlike the previous construct that did

not exhibit allosteric regulation by GTP, ADP, and palmitoyl-CoA, the current construct was sensitive to all regulators tested. In fact, the current antenna-less construct is hypersensitive to activation by both ADP and leucine. Notably, the current antenna-less construct has approximately 10% the basal activity of wild-type GDH. Although the previous antenna-less construct has an unknown basal activity compared to wild-type GDH, the removal of the antenna in the current construct significantly lowers the basal velocity of the resulting protein. Since the antenna is critical for catalysis, not just allostery, the purpose of the antenna's evolution appears to be more fundamental than simply a facilitator of the complex allostery of human GDH. The antenna appears to make catalysis more energetically favorable [40]. As ADP expedites the rate-limiting step of GDH catalysis, product release, the antenna itself seems to work in a similar way. Wild-type GDH has moderate activity and can be very effectively activated and inhibited. Without the antenna present, the basal activity drops substantially, and further inhibition of the enzyme has negligible effect on the flux of metabolites through the GDH pathway. It can be very effectively activated, but the unidirectional modulation of activity does make the enzyme less tunable than wild-type GDH [44].

Since the whole antenna domain is integral to catalysis and proper allosteric regulation, the next step is to determine the contributions of the individual antenna residues to catalysis and allosteric regulation. The residues of interest are clinically relevant HI/HA mutants. The benefits of investigating these antenna residues are twofold. First, understanding the basis of the allosteric dysregulation that results in disease will facilitate the development of comprehensive treatments for HI/HA. Second, from a basic

science point of view, the role of the individual antenna residues to proper allosteric regulation will further our understanding of the role of the antenna as a whole.

Mutation of the residues of the descending helix of the antenna can cause pathological increases in activity in several ways as detailed in chapter 3. First, they can desensitize the enzyme to allosteric inhibition by GTP. Second, mutation of a single residue could make the protein more sensitive to allosteric activation by activators such as leucine or ADP, or third, mutations could cause the protein to have increased basal activity compared to wild-type GDH. It was previously assumed that HI/HA was a result of mutated GDH being less sensitive to allosteric inhibition by GTP. The work presented here demonstrates that mutating residues in the descending antenna helix can hyper-activate the enzyme via other means.

Although none of the residues investigated in this study contact a known allosteric regulator-binding site, all HI/HA mutations examined here do show profound alterations in allosteric regulation and/or basal catalytic activity. In agreement with the previous understanding of what causes pathogenic levels of GDH activity in HI/HA, several of the residues do appear to be significantly less sensitive to GTP inhibition, namely F440L, G446R, and S445L. The other residues investigated, P436L and S448P, also show some slight decrease in sensitivity to GTP, but it is not as profound as the decrease in GTP inhibition found in F440L, G446R, and S445L. Despite P436L and S448P having only a slight decrease in sensitivity to GTP inhibition, they display a significant increase in activity by the second possible mechanism introduced above, they are more sensitive to allosteric activation by both ADP and leucine. The antenna-less mutant discussed in Chapter 2, S448P, and P436L are very different from one another but appear to display

similar characteristics. Whereas the antenna-less mutant is the result of a removal of the antenna, P436L is located at the top of the descending helix of the antenna in a loop that connects it to the ascending helix, and S448P is located at the very base of the antenna in the loop that connects the descending helix to the pivot helix. P436L and S448P are hypersensitive to activation and have significantly decreased basal activities as they operate at 20% and 50% of the basal activity of wild-type GDH, respectively. To further explore possible similarities between P436L and S448P, substrate inhibition experiments need to be conducted on these and the other HI/HA mutations investigated in this study to compare how increasing glutamate concentrations affects the various classes of mutations. The antenna-less construct requires significantly higher glutamate concentrations before the velocity of the reaction begins to be inhibited. As substrate inhibition is closely related to the rate limiting product release step of catalysis, it would be interesting if P436L and S448P displayed an alteration in substrate inhibition similar to the antenna-less mutant. Such a similarity could indicate that these mutants have a different rate-limiting step than product release. The next experiments to confirm what the rate limiting steps are for both the antenna-less construct and specific HI/HA mutants would include direct binding experiments similar to the stopped flow work done by Bell [9]. Bell and colleagues used the spectroscopic properties of the various GDH ternary complexes to determine what forms of coenzyme and substrate were bound to the active site. As the GDH oxidative deamination reaction involves the generation of a Schiff base and hydride transfer, there are multiple steps in the reaction mechanism that can be rate limiting. Using deuterated glutamate, Bell and colleagues were able to determine that, because the burst rate is negligibly affected by deuteride versus hydride transfer, the

chemical transfer step must be faster than some conformational step that precedes it, but product release is the overall rate-limiting step. Previous studies have exploited the unique spectroscopic properties of various GDH-coenzyme-substrate ternary complexes. NAD(P)H is excited at 340 nm and emits at 460 nm. The 340 nm band of the GDH-NADPH-D-glutamate ternary complex is red shifted while the GDH-NADPH-2-oxoglutarate produces a blue shift of the 340 nm band [114]. Since both glutamate and 2-oxoglutarate bind to the same site, the difference in spectroscopic properties of the two ternary complexes is likely due to the realignment of the charged non-polar groups of the enzyme in proximity to the reduced nicotinamide group [114]. Similar investigations should be done with the various mutants discussed here, such as the antenna-less mutant, to more definitively determine if the rate-limiting step of the reaction is altered as the substrate inhibition experiments suggest. To perform these studies, purified protein is required. One of the major issues with the antenna-less construct is that it is highly unstable. A his-tagged version of the antenna-less construct was generated to expedite purification. The his-tagged antenna-less protein is kinetically identical to the untagged version and the his-tag can be removed since it is connected via a rTEV protease cleavage sequence. Although the addition of the his-tag does aid in purification, there are foreseeable difficulties to overcome to generate the amount of purified protein required to perform the stopped-flow experiments.

A major goal of future studies should be to determine the structure of the antenna-less protein. Determining the structure of the antenna-less GDH could provide evolutionary insight into the role of the antenna as various bacterial, ciliate, and animal forms of GDH and their respective antennas can be compared. Importantly,

understanding the structure of the protein could lead to a better understanding of how the hyper-activation of ADP and leucine is occurring. Additionally, a structure of the antenna-less GDH would open the door to computational studies to understand the binding free energy differences in ligands or conformational differences between subunits as has been previously performed with wild-type GDH [113]. Mutants like P436L that have the his-tag added have proven much easier to purify and could represent a much more feasible starting point for stopped flow experiments to confirm the rate limiting step of the reaction and to perform structural investigations.

Overall, these results refined the field's understanding of the role of the antenna domain of GDH and demonstrated that it is critical not only for proper allosteric regulation, but for catalysis as well. The individual residues of the antenna and their individual contribution to the enzyme's function are as complex as the antenna itself. Insights gained into the properties of these various clinical mutants are highly significant as it demonstrates that pathogenic hyperactivity caused by single mutations to the GDH antenna can disrupt regulation by numerous regulators, not just desensitize the enzyme to GTP inhibition as previously thought. Future work should build on these studies to further elucidate the role of the antenna in the complex allosteric regulation of GDH. Insights gained through structural studies and computational analysis can then be used to develop experiments and interpret results in much more complex systems *in vivo.*

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Vita

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