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ORIGINAL PAPER

Glutamate Dehydrogenase, a Complex Enzyme at a Crucial Metabolic Branch Point

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Abstract In-vitro, glutamate dehydrogenase (GDH) catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate (α-KG). GDH is found in all organisms, but in animals is allosterically regulated by a wide array of metabolites. For many years, it was not at all clear why animals required such complex control. Further, in both standard textbooks and some research publications, there has been some controversy as to the directionality of the reaction. Here we review recent work demonstrating that GDH operates mainly in the catabolic direction in-vivo and that the finely tuned network of allosteric regulators allows GDH to meet the varied needs in a wide range of tissues in animals. Finally, we review the progress in using pharmacological agents to activate or inhibit GDH that could impact a wide range of pathologies from insulin disorders to tumor growth.

Keywords Glutamate dehydrogenase · Allostery · Insulin

Introduction

Glutamate dehydrogenase (GDH) is found in all living organisms and catalyzes the oxidative deamination of L-glutamate to α-KG using $NAD(P)^+$ as a coenzyme (Fig. 1) [1]. This homohexameric mitochondrial enzyme has subunits comprised of ~500 amino acids in animals. GDH from animals, but not other kingdoms [2], is allosterically regulated by a wide array of ligands (Fig. 2) [2–8]. Under most conditions, the rate limiting step for the enzyme is product release and allosteric regulation is mediated by controlling this step. GTP [8–10], and with ~100-fold lower affinity, ATP [2], are inhibitors of the reaction and act by increasing the binding affinity for the product (Fig. 1), thereby decreasing enzymatic turnover [10]. Palmitoyl CoA [11], steroid hormones [12], and diethylstilbestrol [4] (DES) are a group of hydrophobic and potent inhibitors. ADP is an activator of GDH [2, 5, 9, 10, 13] 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 [7]. Leucine activation is akin to ADP but is believed to act at a site distinct from ADP [14]. Perhaps most importantly, these allosteric regulators interact in agonistic and antagonistic ways. NADH binding to a non-catalytic site enhances GTP binding and visa-versa [8, 10]. ADP [5, 15] and leucine [16, 17] have strong antagonistic effects on GTP binding and inhibition. ADP and NADH directly compete for the same binding site [8]. Finally, GDH is also regulated by other mitochondrial enzymes; SCHAD [18] and SIRT4 [19]. Therefore, GDH activity in-vivo is finely tuned by the balance of, and interplay between, all of these allosteric regulators rather than just being turned off and on by inhibitors and activators.

There is a second form of GDH, GDH2, that is only found in humans and apes [20]. GDH2 is an intron-less form of GDH that is thought to arise from retroposition of GDH1 [20] less than 23 million years ago [21]. GDH2 is X-linked and found mainly in neuronal and testicular tissue [20]. GDH2 is quite different than GDH1 in that it is less sensitive to GTP inhibition but more sensitive to ADP activation, is much less thermostabile, and has a lower activity than GDH1. In fact, GDH2 activity requires ADP activation [22].

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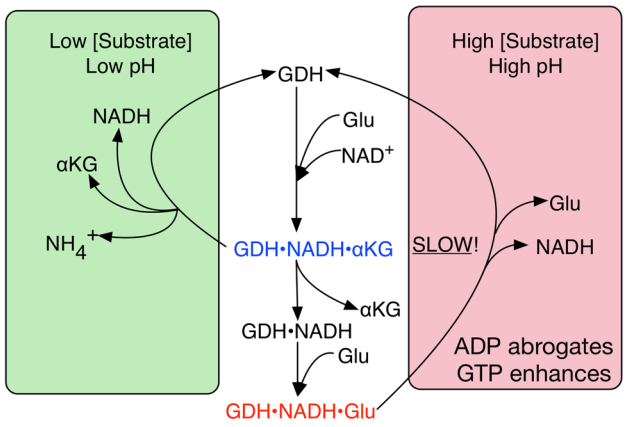

Fig. 1 Schematic diagram of glutamate dehydrogenase catalysis. Under high glutamate concentrations, glutamate replaces α-ketoglutarate before reduced coenzyme is released, forming an abortive complex. This is slowly resolved and the next catalytic cycle is started (red box). At lower concentrations of substrate (green box), the abortive complex is not formed and the enzyme is rapidly recycled for the next round of catalysis. The GDH·NADH·aKG and the GDH·NADH·Glu complexes are colored blue and red, respectively, to note that the reduced coenzyme in these complexes can be directly observed as blue and red shifted species in pre-steady state stopped-flow analysis. As also noted in the red box, GTP and ADP stabilize and destabilize this abortive complex, respectively. (Color figure online)

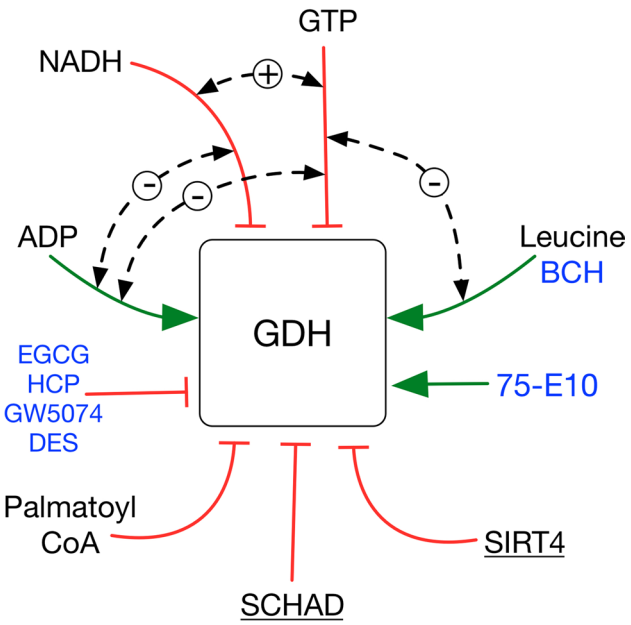

Fig. 2 Regulation of GDH. The red and green lines represent inhibition and activation, respectively. The compounds shown in blue represent synthetic allosteric regulators. The dashed lines represent the antagonism or agonism between the various allosteric regulators. SCHAD and SIRT4 are not allosteric ligands but rather enzymes that interact with GDH and cause inhibition. (Color figure online)

As with GDH1 [23], GDH2 is mainly localized to the mitochondria. A small fraction of GDH1 and GDH2 has been shown to be also associated with the endoplasmic reticulum, but in an unprocessed form with the leader sequence intact [24]. The metabolic role, if any, of the cytoplasmic form of GDH remains unclear.

There is growing evidence that GDH might play important roles in CNS development and pathologies. From sequencing GDH1 and GDH2 genes in Parkinson's disease patients, an A445S mutation in GDH2 that increases activity was found to be correlated with 6–13 year earlier onset of symptoms [25]. Presumably because this variant is more sensitive to estrogen inhibition, this phenotype was only observed in males. Transgenic animals over-expressing neuronal GDH1 exhibit age-dependent degeneration of the CA1 hippocampal region similar to Alzheimer's disease pathology [26]. Therefore, proper regulation of GDH is clearly required for healthy brain function.

The Atomic Structure of GDH

The structure of GDH (Fig. 3) is a trimer of dimers with each subunit being composed of at least three domains [27–30]. The bottom domain makes extensive contacts with the adjacent subunit from the other subunit in the dimer. Resting on top of this domain is the 'NAD binding domain' that has the conserved nucleotide-binding motif. Animal GDH has a long protrusion, 'antenna', rising above the NAD binding domain that is not found in bacteria, plants, fungi, and the protists other than the *Ciliates* [31]. The antenna from each subunit lies immediately behind the adjacent, counter-clockwise neighbor within the trimer. Since these intertwined antennae are only found in animal and *Ciliate* GDH that is allosterically regulated, we initially speculated that it likely played an important role in regulation [27].

GDH Dynamics

From the structures of GDH with and without active site ligands, it was possible to infer the conformational changes that occur throughout the hexamer during each catalytic cycle [27–30]. When substrate is released from the deep recesses of the active site cleft, the coenzyme binding domain rotates up by ~18° and the antenna domain in each subunit rotates counter clockwise subunit within each trimer. As the catalytic cleft opens, the base of each of the long ascending helices in the antenna appears to rotate out in a clockwise manner as the pivot helix of the adjacent subunit is pushed back. This motion gives the antenna a clockwise motion. The short, distended helix in the descending loop of the antenna coils back into a longer, better ordered helix

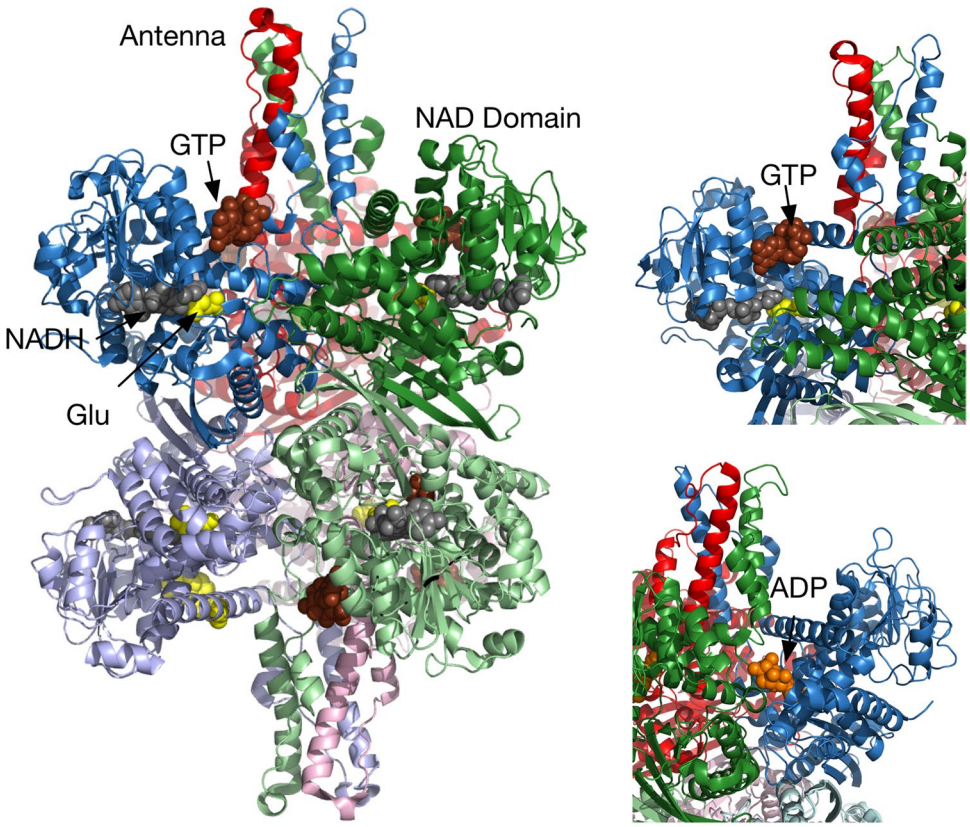

Fig. 3 Structure of glutamate dehydrogenase. The image on the left is of the entire GDH hexamer with each subunit shown in different colors. Glutamate (yellow) and NADH (grey) bound to the active site are shown as space filling models. The inhibitor, GTP, is shown as a brick colored space filling model. The images on the right show close up views of GTP and ADP bound to the enzyme. GTP binds between the NAD binding domain and the antenna whereas ADP binds behind the NAD^+ binding domain. (Color figure online)

akin to releasing an extended spring. Finally, the core of the entire hexamer seems to expand as the mouth opens. The three pairs of subunits that sit on top of each other move as rigid units away from each other, opening the cavity at the core of the hexamer.

The GTP Inhibition Site

GTP is a potent allosteric inhibitor for the reaction and binds at the base of the antenna, wedged between the NAD binding domain and the pivot helix [27, 28] (Fig. 3). It is important to note that this binding site is only available for GTP binding when the catalytic cleft is closed. Therefore, it is likely that GTP binds to the 'closed' conformation and makes it more difficult for the 'mouth' to open and release product. This is entirely consistent with the finding that GTP inhibits the reaction by slowing down product release by increasing the binding affinity of substrate and coenzyme (Fig. 1) [9, 10, 32]. The vast majority of the interactions between GTP and GDH involve the triphosphate moiety with the majority of the salt bridges being made with the γ-phosphate, explaining why GTP >> GDP > GMP with regard to inhibition [2]. Therefore, when the mitochondrial energy level is high, (high GTP and ATP levels) GDH will be inhibited.

The ADP/Second NADH Site Paradox

Perhaps one of the most confusing regulatory sites on mammalian GDH is the allosteric activator, ADP, binding site. To this site, NAD(H) and epigallocatechin gallate, (EGCG) and epicatechin gallate (ECG) also bind but inhibit rather than activate the enzyme (Fig. 3). The existence of a second NADH binding site per subunit was demonstrated both kinetically and by binding analysis [3, 33, 34]. It was observed that NADH alone binds with a stoichiometry of 7–8 molecules per hexamer. In the presence of glutamate, NADH binds more tightly and the stoichiometry increases to 12 per hexamer [34]. Similarly, GTP also increases the affinity and binding stoichiometry [10]. This second coenzyme site strongly favors NADH over NADPH with Kd's of 57 and 700 μM, respectively. In the case of oxidized coenzyme, NAD^+, two binding sites were also observed. While the recent structures of the various complexes have demonstrated that ADP and NAD(H) bind to the same site [28, 30], this was first suggested by ADP binding competition with NAD^+ [35] and NADH [8]. However, before the structures were determined, it was never clear whether this competition was due to steric interference or allosteric effects of ADP. These binding studies provided direct evidence that GTP and glutamate enhance binding of NADH to a second

site and ADP blocks binding of both NAD^+ and NADH to a second site. The typical cellular concentrations of NADPH are well below the Kd for this second binding site while those of NADH are in the range of the observed Kd for the second site.

There appears to be, however, a disconnect between NADH binding to this site and NADH inhibition. In the absence of other allosteric regulators, NADH inhibition is observed at concentrations above 0.2 mM (e.g. see [36]), but only reaches ~ 50% inhibition at 1 mM NADH. Importantly, GTP enhances the binding of NADH to this second site by about fivefold (~ 9.7 µM), putting the Km well below the expected cellular concentration. Therefore, perhaps NADH does not inhibit alone but rather is designed to work synergistically with GTP regulation; under conditions of high reductive potential, NADH acts with GTP to keep GDH in a tonic state. If NADH binding plays any role in GDH regulation, then it is possible that this regulation offers a feedback mechanism to curtail glutamate oxidation when catabolic reductive potentials (NADH) are high. However, since the Kd for NADPH is so high, it appears to not sense anabolic reductive potential.

ADP Binding Site

While NADH and ADP bind to the same allosteric site, they have quite different effects on the enzyme. In the oxidative deamination reaction, ADP activates at high pH, but inhibits at low pH with either NAD^+ or $NADP^+$ as coenzyme [5]. In the reductive amination reaction, ADP is a potent activator at low pH and low substrate concentration. At pH 6.0, high concentrations of α-KG and NADH, but not NADPH, inhibit the reaction. This substrate inhibition is alleviated by ADP [5]. Therefore, while GTP and glutamate bind synergistically with NADH to inhibit GDH, ADP activates the reaction by decreasing the affinity of the enzyme for coenzyme at the active site. Under conditions where substrate inhibition occurs, this activates the enzyme. However, under conditions where the enzyme is not saturated (e.g. low substrate concentrations), this loss in binding affinity causes inhibition. Therefore, under conditions where product release is the rate-limiting step, ADP greatly facilitates the catalytic turnover. It should be noted that the fact that substrate (α-KG) inhibition in the reductive amination reaction is only observed using NADH as coenzyme was suggested to be due to NADH (but not NADPH) binding to the second coenzyme site. Further, it was suggested that ADP activation under these conditions was due to ADP displacement of NADH from the second allosteric site [2].

Leucine Activation

Leucine, as well as some other monocarboxylic acids, has been shown to activate mammalian GDH by facilitating coenzyme release in a manner similar to ADP but probably not via the same binding site [14]. Since leucine is a weak substrate for GDH, one binding site is the active site. It is unclear whether there is a second, allosteric, leucine-binding site and its possible location is not known. The choice of leucine as a regulator is likely not accidental since leucine is the most abundant amino acid in protein (10%). Leucine levels, therefore, provide a good metric of protein abundance. Therefore, leucine serves as an effective signal to GDH that there has been pulse of free amino acids from protein digestion.

To date, we have not been able to visualize the leucine bound to the activation site on animal GDH. This is more than likely due to the high (mM) ED_{50} for leucine and the very high concentrations of solutes necessary for GDH crystallization. However, structural studies on GDH from an extremely thermophilic bacteria, *Thermus thermophiles*, have suggested a possible location for the leucine activation site [37]. This bacterial form of GDH is rather different than animal GDH in that it has two different types of subunits; a regulatory subunit, GDHA, and a catalytic subunit, GDHB. Leucine can activate the wild type of this GDH by ~ 10-fold. The structure heterohexameric GDH complexed with leucine showed leucine bound to the subunit interface not far from the core of the enzyme and this was confirmed via site-directed mutagenesis. Since there is significant homology between the bacterial and animal GDH, it is possible to estimate a possible location of the bound leucine in animal GDH by overlaying the bacterial and animal structures (Fig. 4). As noted by the authors, leucine appears to bind in the equivalent region to which our inhibitors, bithionol and GW5074, bind in animal GDH (as reviewed below). Interestingly, this dimer interface expands and contracts during catalysis and is not far from the ADP activation site. Could ADP and leucine be activating GDH by facilitating this motion?

Metabolic Role of GDH

For years, GDH was relegated to a seemingly unimportant role as 'housekeeping' enzyme because of its high expression levels and suggestions that the enzyme functions in equilibrium. This view conflicts with animal GDH with its extensive allosteric regulation. Allosteric regulation strongly suggests that GDH is not working in equilibrium nor is it just an amino acid catabolism pipeline. If the enzyme is operating in both enzymatic directions, allosteric activation and/or inhibition would have little effect on the final

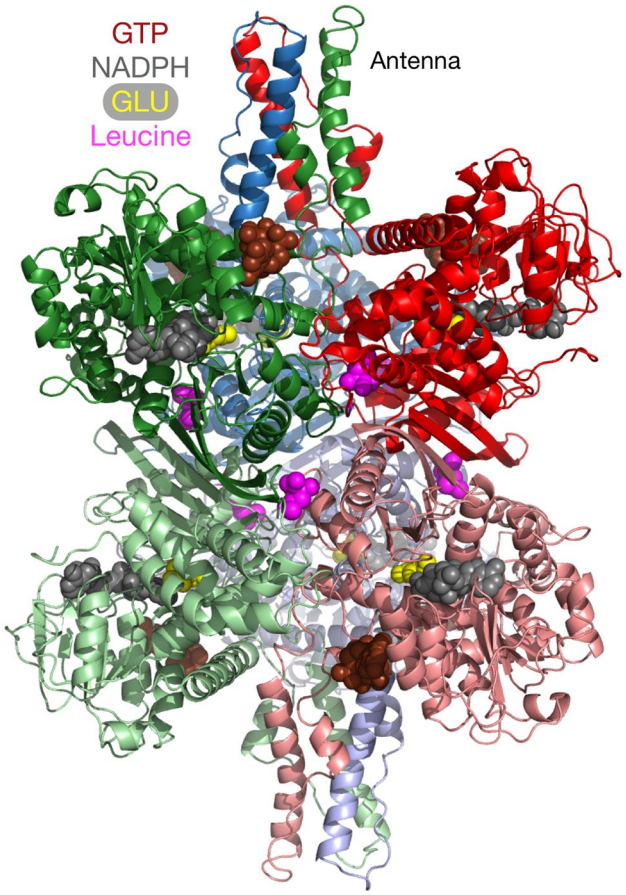

Fig. 4 Possible location of the leucine activation site. The structure of GDH from *Thermus thermophiles* complexed with the activator leucine [37] was aligned with bovine GDH. Shown here in mauve is the location of the bound leucine from the bacterial structure to suggest a possible location for the leucine activation site. (Color figure online)

metabolite levels, just the rate at which equilibrium is established. Allosteric regulators are used on enzymes that are not operating at near-equilibrium and affect flux has been previously discussed [38]. GDH is an excellent example of this with regard to ADP and GTP regulation. GTP can inhibit the reaction by more than 95%. While ADP alone can only activate GDH by about twofold, it can nearly remove GTP inhibitory effects. In this way, by increasing the ADP/GTP ratio, the mitochondria can experience a rapid burst in glutamate catabolism by several orders of magnitude.

There has been some controversy with regard to the reaction directionality of GDH and this is relevant to whether GDH is in equilibrium in-vivo. A review of numerous studies make it fairly clear that GDH mainly catabolizes glutamate. One way to understand the metabolic role of GDH is by reviewing the physiological levels of metabolites and GDH Km's for these substrates (Table 1). It is important to note that the following discussion is only meant as a general

Table 1 Kinetic parameters for bovine GDH

Metabolite	Km at pH 6.0	Km at pH 8.0
Oxidative deamination		
Glutamate	10 mM [5]	3 mM [5]
NAD^+	0.2 mM [5]	0.01 mM [39][a]
$NADP^+$		0.05 mM [40][a]
Reductive amination		
a-KG	30 mM [5]	0.3 mM [5]
NADH	0.002 mM [5]	0.02 mM [5]
NADPH		0.03 mM [40]
NH_4^+	164 mM [5]	13 mM [5]

[a]Approximate Km values determined at high coenzyme concentrations since negative cooperativity is apparent over the wider range of concentrations

discussion of approximate metabolite levels with respect to GDH kinetic efficiencies, with the main conclusion being that the extremely high Km that GDH has for ammonium makes it highly unlikely that GDH synthesizes glutamate in-vivo. All of the metabolites vary in concentration between the various tissues and within the tissues themselves.

The reported levels of oxidized and reduced coenzyme vary significantly, but approximate levels can be estimated. In mammalian mitochondria, assuming a matrix volume of 1 µl/mg of protein, the concentrations of NAD(H) and NADP(H) are approximate 0.5–2.0 mM [41]. However, activity of the transhydrogenase transfers much of the reductive power of NADH to NADPH. Using metabolite indicators, the mitochondrial NADH/NAD^+ ratio was estimated to be ~0.2 and the NADPH/$NADP^+$ ratio was ~200 [42]. The total amount of NAD(H) in the cell is about tenfold higher than NADP(H) [43]. Taken together, the approximate level of NAD^+ is 0.4–1.7 mM, NADH is 0.08–0.3 mM, $NADP^+$ is 0.0002–0.01 mM, and NADPH is 0.05–0.2 mM. Therefore, NAD^+ is found in the highest concentration while NADH and NADPH should be at comparable concentrations. Obviously, these levels are prone to change depending upon the metabolic state of the mitochondria. Reported cellular levels of glutamate and α-ketoglutarate (α-KG) suggest that glutamate levels are higher than α-KG. In the mitochondria, the concentration of glutamate is ~17 mM [44] whereas α-KG is ~1.6 mM in the mitochondria and ~0.3 mM in the cytoplasm [45]. Normally, ammonium serum levels are less than 0.05 mM.

So, how do these physiological levels compare to the kinetic constants and what does it tell us about the directionality of GDH in-vivo? While this greatly simplifies the complexity of substrate levels in various tissues and cell types, it is still an interesting exercise to develop a general picture of GDH function. With both glutamate and α-KG, the physiological concentrations are well within the In-vitro

Km values and do not suggest a preference. It is interesting, however, that substrate binding is better at high pH for oxidative deamination and the better at low pH in the reductive amination reaction [5]. Among the various forms of coenzyme, only levels of $NADP^+$ are lower that its In-vitro Km. Of the remainder, the levels of NAD^+ are the highest with respect to its Km. This is consistent with quantitative enzyme histochemical analysis that demonstrated that NAD^+ is used ~ 2.5 fold more than $NADP^+$ for GDH catabolism [46]. The greatest Km and concentration disparity is with regard to ammonium. The Km of ammonium is ~ 5000 fold higher than the typical serum levels of ammonium [5]. Put another way, less than 1 out of 5000 GDH molecules are expected to be bound with ammonium under physiological conditions while more than half of GDH can be expected to be bound with NAD^+ and glutamate. Further, NH_3 can freely diffuse across the mitochondrial membrane and the intracellular concentration of ammonium is roughly the same as the extracellular (e.g. [47, 48]). For these reasons alone, it is very hard to argue that GDH favors the reductive amination reaction.

The strongest evidence for GDH acting in the oxidative deamination reaction comes from numerous in-situ and in-vivo metabolite studies. The hyperinsulinism/hyperammonemia (HHS) disorder is caused by a loss of GTP inhibition that leads to a marked increase in ammonium production and increase insulin secretion presumably by feeding α-KG into the Krebs cycle. Stable isotopic analysis of wild type GDH showed that activation by leucine increased the flux of glutamate through GDH threefold [49]. However, in the presence of high glucose that increases the GTP/ADP ratios, GDH was inhibited to such a degree that leucine could no longer activate glutamate catabolism [49]. When the HHS (H454Y) form of GDH was expressed in the pancreatic tissue of mice, an enhanced GDH activity and lowered GTP sensitivity was observed [50]. $^{15}NH_4Cl$ tracer studies directly demonstrated that ^{15}N was not incorporated into glutamate in either the H454Y transgenic or in normal islets. Similar studies have shown that neurons can seamlessly switch from glucose to glutamate metabolism [51], therefore showing that glutamate catabolism is favored in-vivo. Extensive isotope tracer studies have also shown that, in the brain, GDH is not a major route for ammonia removal even under hyperammonemia conditions [52]. Indeed, even when glutamine synthetase is inhibited with L-methionine-S,R-sulfoximine (MSO), flux of ammonium to glutamate via GDH is about 4% relative into glutamine compared to untreated rats. Studies on the brain have also demonstrated that even under hyperammonemic conditions, GDH operates in the oxidative deamination direction [53, 54]. These same conclusions about GDH directionality have been reached by other groups as well (e.g. [55]). Interestingly, even in plants, ^{15}N incorporation studies in the presence of excess ammonium have shown that GDH functions in the oxidative deamination direction [56].

Finally, to review, there are much more efficient pathways to clear ammonium and generate glutamate than via GDH-mediated reductive amination of α-KG. For example;

Glutamine synthetase:

$$NH_4^+ + \text{glutamate} + ATP \rightarrow \text{glutamine} + ADP + Pi$$

Glutamate synthase:

$$\text{glutamine} + \alpha\text{-KG} + NADPH \rightarrow 2 \times (\text{glutamate}) + NADP^+$$

This pathway for creating glutamate has two major advantages over GDH. Firstly, the Km for ammonium with glutamine synthetase (GS) is far lower (more than 100-fold) than with GDH [57]. Secondly, since GS is an ATP dependent enzyme, it effectively acts to remove ammonium in a somewhat irreversible manner. This could also affect the GDH reaction by pulling it towards the oxidative deamination reaction. Therefore, the overwhelming consensus is that GDH operates in the oxidative deamination reaction. It is important to note that this highly simplified discussion is mainly meant to show a more efficient way to create glutamate without GDH to address the directionality of the GDH reaction. However, the whole process of glutamate oxidation in-vivo is far more complicated and highly tissue dependent but still is dominated by GDH (e.g. [58]).

The Role of GDH in Insulin Homeostasis

For many years, it was not at all clear why animal GDH exhibited such complex regulation. Then, using a nonmetabolizable analog of leucine, BCH (β-2-aminobicyclo(2.2.1)-heptane-2-carboxylic acid) [6, 59] it was suggested that this regulation may play a role in insulin homeostasis. In the pancreatic β-islets, this leucine mimic increases GDH activity leading to increase of glutamate catabolism that, in turn, increases the ATP/ADP ratio in the cell. The elevated ATP levels close the plasma membrane K_{ATP} channels, depolarizes the membrane potential, opens voltage gated Ca^{2+} channels, and causes insulin granule exocytosis. The clearest evidence that GDH likely plays an important role in insulin homeostasis came from the discovery that the hyperinsulinemia/hyperammonemia (HHS) syndrome is caused by loss of GTP regulation of GDH [60–62]. Patients with HHS have increased β-cell responsiveness to leucine and susceptibility to hypoglycemia following high protein meals [63]. This is likely due to uncontrolled amino acid catabolism, yielding high ATP levels that stimulate insulin secretion as well as high serum ammonium levels. During glucose-stimulated insulin secretion in normal individuals, it has been proposed that the generation of high energy phosphates inhibits GDH and promotes conversion of glutamate to glutamine, which,

alone or combined, might amplify the release of insulin [49, 64].

From these and other results, we have proposed an overall picture for GDH regulation in-vivo [65]. GDH is activated when amino acids (protein) are ingested to promote insulin secretion and appropriate anabolic effects on peripheral tissues; in the glucose-fed state, GDH is inhibited in pancreas perhaps to redirect amino acids into glutamine synthesis in order to amplify insulin release. Similarly, adjustment of hepatic GDH allows amino acid degradation to be suppressed when fatty acids and carbohydrates are available, but to be increased when protein (amino acids) are ingested and surplus amino acids can be oxidized. The evolutionary choice of leucine as a regulator is likely because leucine is one of the most abundant amino acids in protein (10%) and provides a good measure of protein catabolism. The marked sensitivity of GDH for GTP over ATP likely serves as a more accurate sensor for the metabolic state of the mitochondria. Most of the ATP in the mitochondria is produced from oxidative phosphorylation that is driven by the potential across the mitochondrial membrane created by NADH oxidation. Therefore, the number of ATP molecules generated from one turn of the TCA cycle can vary between 1 and 29. In contrast, one GTP is generated per turn of the TCA cycle and therefore, with the slow mitochondria/cytoplasm exchange rate, the GTP/GDP ratio is a much better metric of TCA cycle activity than the ATP/ADP ratio. Indeed, recent results demonstrate that mitochondrial GTP, but not ATP, regulates glucose-stimulated insulin secretion [66]. This is consistent with the HHS disorder in that, without GTP inhibition of GDH, glutamate will be catabolized in an uncontrolled manner, the TCA cycle will generate more ATP, and more insulin will be released.

The Role of GDH in Tumor Growth and Neuronal Development

There has been growing interest in the role of GDH in maintaining the high metabolism found in transformed cells. Using ^{13}C NMR spectroscopy, DeBerardinis et al., found that the TCA cycle was still active and the associated substrates were mainly being used for fatty acid synthesis using the reductive power and lactate generated from glutaminolysis [67]. Importantly, most of the amino groups from glutamine were lost from the cell while the carbon backbone combined with TCA intermediates for biosynthetic reactions. Similarly, GDH was found to be necessary for glioblastoma cells to survive impaired glucose metabolism or Akt signaling [68]. They showed that if GDH activity was impaired, the cells were still able to utilize glucose. However, if the cells were deprived of glucose or if glucose metabolism was impaired, then the cells absolutely required GDH activity for growth. Recent studies have also shown how other mitochondrial enzymes play a key role in regulating GDH-mediated glutaminolysis [69]. These studies demonstrated that activation of mammalian target of rapamycin complex 1 (mTORC1) enhances glutaminolysis by repressing the sirtuin, *SIRT4*, that acts to inhibit GDH activity. When *SIRT4* is overexpressed, cell proliferation, tumor development, and transformation are all inhibited. Finally, SIRT4 and GDH have been shown to play antagonistic roles in glial cell development with the hyperactive HHS form of GDH accelerating glia development while overexpression of SIRT4 inhibiting gliogenesis [70]. Finally, the enhanced GDH activity observed in tumors has been shown to be a possible prognostic marker in colorectal cancer patients and indicator of metastasis [71]. All of these studies directly demonstrate that GDH operates in the oxidative deamination and its regulation is pivotal in the regulation of cell proliferation and development.

There is also evidence that GDH works with other pathways involved in oncogenesis. For example, somatic mutations in isocitrate dehydrogenase (IDH) occur in 70–90% of low grade glioma [72] and secondary glioblastoma multiforme [73]. These mutations cause IDH to reduce isocitrate to D-2-hydroxyglutarate rather than α-ketoglutarate. D-2-hydroxyglutarate is an oncometabolite that inhibits dioxygenases and α-ketoglutarate-dependent DNA and histone demethylases. The loss of normal IDH activity causes a loss in α-ketoglutarate and reduced coenzyme. This is compensated for by increased expression of GDH1 and GDH2 but inhibition of GDH2 expression in these glioma cells slows cell growth [74]. Further, isotope labeling studies have shown that glutamate catabolism via GDH2 in glioma progenitor cells promotes lipid biosynthesis [74]. These results clearly demonstrate that, not only does GDH play a critical role in tumor growth, but clearly GDH operates in the oxidative deamination direction.

Regulation by Other Mitochondrial Enzymes

Short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) belongs to the short chain dehydrogenase/reductase superfamily and acts on a wide spectrum of substrates, including steroids, fatty acids, and cholic acids, but prefers short chain methyl-branched acyl-CoAs. Patients born homozygous deficient in the enzyme medium/short chain SCHAD (M/SCHAD) have symptoms akin to HHS with hyperactive GDH and sensitivity to leucine, glutamine, and alanine. From our transgenic *hadh* -/- mouse studies, we demonstrated that our GDH inhibitors discussed below can control this disorder In-vitro [18].

SIRT4 is a mitochondrial enzyme that uses NAD^+ to ADP-ribosylate and inhibit GDH activity [19]. Loss

of SIRT4 in insulinoma cells and β-cells from SIRT4 deficient mice activates GDH that, in turn, upregulates GDH-mediated insulin secretion. Interestingly, a similar effect is observed in pancreatic β-cells from on a calorie restricted diet. Since the GDH from these mice was insensitive to phosphodiesterase, it was concluded that the GDH was not ADP-ribosylated. These results indicate that SIRT4 represses GDH (and subsequent GDH-mediated insulin secretion) via ADP-ribosylation and these effects that are abrogated during calorie restriction. This is yet another example of GDH being activated when the mitochondria is at a low energy state.

The Search for Therapeutic GDH Inhibitors

While the hypersecretion of insulin can be controlled with compounds such as diazoxide [75], it does not treat the serum ammonium and CNS pathologies. High throughput screening was used to find new inhibitors of GDH to control the dysregulated GDH in HHS [39]. These lead compounds have quite disparate chemical properties and therefore it seemed more than likely that the various classes were binding to different sites.

The Structure of Hexachlorophene (HCP) Bound to GDH

In the GDH-HCP complex [76], six molecules of HCP form a ring in the inner cavity of the hexamer [76]. The majority of the interactions between HCP and GDH are hydrophobic and form a ring of stacked aromatics. HCP binds in two orientations. In the first, the rings of HCP approximately stack against two Y190 sidechains from diagonally adjacent subunits. HCP in the other binding orientation makes hydrophobic interactions with M150, I187, Y190 and the methylene side chain atoms from T186 and K154. In addition, the aromatic rings of the HCP molecules stack against each other in this ring conformation.

Biothionol and GW5074 Complex Structures

Bithionol and GW5074 bind in essentially the same site that is distinct to the HCP site (Fig. 5a, b) [76]. These two drugs bind halfway between the core and the exterior of the hexamer. Residues 138–155 of the glutamate-binding domain form an α-helix that makes most of the contact between diagonal subunits and draw closer together when the catalytic cleft is closed. These two drugs stack against each other and interact with hydrophobic residues and the aliphatic portions of the polar and charged side chains of residues K147, R150, R151, and R150 (Fig. 5c). These compounds,

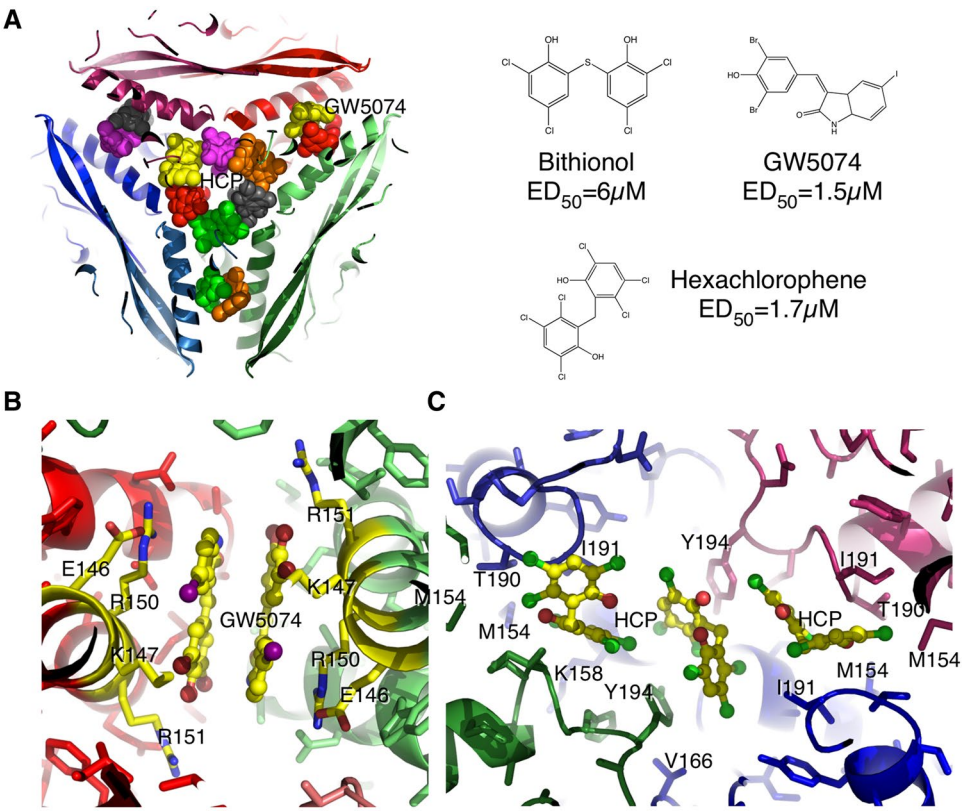

Fig. 5 Locations of three compounds that inhibit GDH activity. **a** Shows a wedge of the GDH core, viewed down the threefold axis, with the bound compounds represented as space filling models. Bithionol and GW5074 bind to the same site, between adjacent subunits, and midway between the core of the enzyme and its exterior. Hexachlorophene binds as a ring in the core of the enzyme. **b** Shows how pairs of GW5074 stack against each other and lie between adjacent GDH subunits. **c** Shows the aromatic stacking that allows hexachlorophene to form the ring structure in the GDH core

therefore, appear to directly bind to the area that compresses during mouth closure. As noted above, structural studies on GDH from an extremely thermophilic bacteria, *Thermus thermophilus*, suggest that this bithionol/GW5074 binding site might be involved in leucine allosteric activation [37]. While these compounds are inhibitors rather than activators like leucine, the effects of all of these compounds point to this region being an important control point in structural dynamics that are necessary for enzymatic activity.

Structure of ECG Bound to GDH

From some older papers on diabetes (e.g. [77]), we supposed that the polyphenols from green tea might affect GDH activity. Green tea is a significant source of a type of flavonoids called catechins: including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). One 200 ml cup of green tea supplies 140, 65, 28, and 17 mg of these polyphenols, respectively [78]. EGCG has been suggested to decrease cholesterol levels [79], act as an antibiotic [80] and anticarcinogen [81], repress hepatic glucose production [82], and enhance insulin action [83]. The exact mechanism of action of EGCG with regard to these various effects is largely unknown and in many cases, is assumed to be due to its antioxidant activity. From both isolated studies on purified catechins and then from HTS, we found that both EGCG and ECG were potent inhibitors of GDH [84].

We determined the structure of the GDH/ECG complex and found that the binding site for EGCG/ECG is quite different than the previous three compounds (Fig. 6). Rather surprisingly, these compounds bind to the ADP activation site [85]. ECG/EGCG are extremely hydrophilic and ECG interactions with GDH are dominated by polar interactions [85]. While the other inhibitors were able to bind to GDH in the closed conformation, ECG appears to have pushed the structural equilibrium towards the open conformation in spite of the presence of high concentrations of Glu and NADPH. Indeed, ECG was never observed bound to GDH in the closed conformation even when the crystals were soaked in very high concentrations of ECG [85]. This is the same as what was observed when crystallizing the ADP/GDH complex [30].

Our previous studies demonstrated that a single mutation (R463A) on the pivot helix abrogated ADP activation without affecting ADP binding, as per TNP-ADP binding [30]. From this we suggested that ADP might be facilitating enzymatic turnover by decreasing the energy required to open the catalytic cleft [30]. Mutagenesis studies on the ECG/EGCG binding site suggest it may be more complicated than that (Fig. 6) [85]. The guanidinium group of R90 stacks up against the aromatic rings in both ECG [85] and ADP [30]. This interaction is likely essential for both regulators since the R90S mutation essentially eliminated polyphenol inhibition as well as ADP activation. This mutation, however, does have some effect on GTP inhibition even

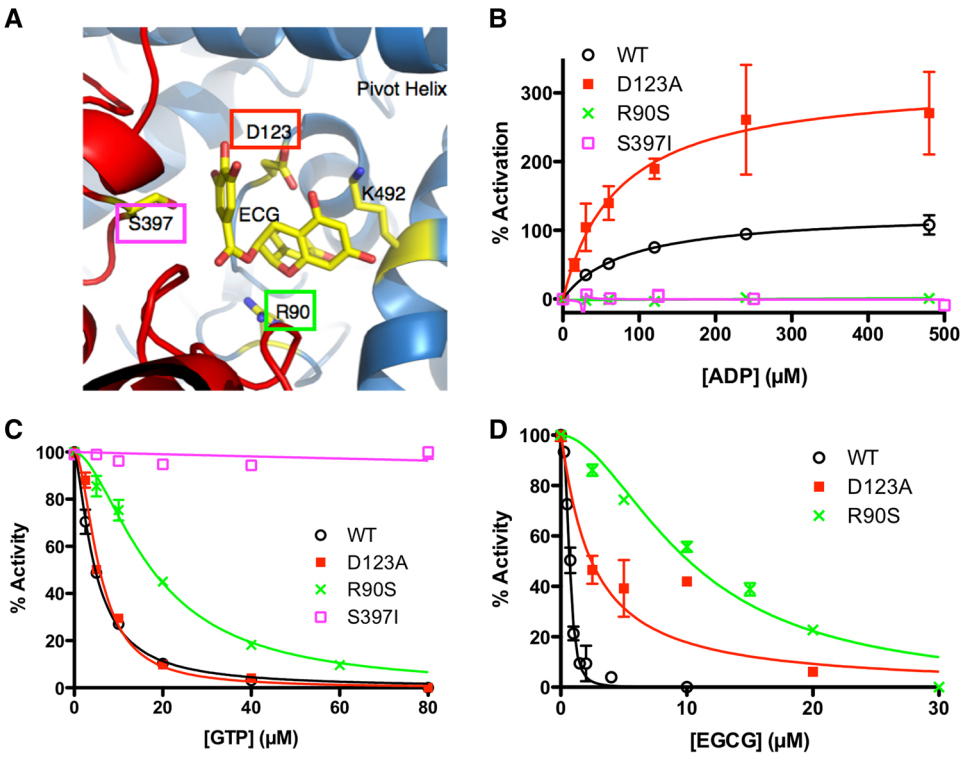

Fig. 6 The binding location of ECG (and EGCG) on GDH. **a** Shows the location of bound ECG which is essentially the same as the ADP site. **b–d** Show the effects of mutating residues in contact with ECG on allosteric regulation by ADP, GTP, and EGCG (Figures adapted from [85])

though R90 is quite distal to the GTP site (Fig. 6). This may be due to the fact that R90 hydrogen bonds to a loop in the adjacent subunit that lies immediately beneath the GTP binding site. D123 lies beneath the pivot helix and hydrogen bonds with the ribose ring on ADP and with a phenolic group on ECG. From this location, it is not surprising that the D123A mutation had no effect on GTP inhibition but did affect polyphenol inhibition. What is surprising, however, is that this mutation actually accentuated ADP activation without significantly affecting its Kact. This may be due to the interactions between D123 and R463. These two side chains form a salt bridge and D123 may shield some of the charge on R463. By removing D123, the R463 interaction with the β-phosphate on ADP may be strengthened and therefore improve the ability of ADP to open the catalytic cleft. This is essentially the opposite of the R463A mutation that abrogates ADP activation by eliminating the charge interaction between R463 and ADP [30]. S397 lies at the base of the antenna and the S397I mutation greatly destabilizes the enzyme while abrogating both GTP and ADP regulation. This may simply be due to the marked sensitivity of the antenna region as exemplified by the fact that removing the antenna also eliminates GTP and ADP activation [31].

Possible Therapeutics for GDH-Mediated Hyperinsulin Disorders

Because of the low ED_{50}'s and their non-toxic nature, a major focus was placed on measuring the effects of the polyphenols on GDH in tissue and in-vivo [85]. Since EC or EGC were not active against GDH, but have the same anti-oxidant activity as ECG and EGCG, the anti-oxidant property of these catechins cannot be relevant to GDH inhibition [84]. Activity, presumably binding, is dependent upon the presence of the third ring structure, the gallate, on the flavonoid moiety. EGCG and ECG allosterically inhibit purified animal GDH In-vitro with a nanomolar ED_{50} [84]. EGCG inhibition is non-competitive and, similar to GTP inhibition, is abrogated by leucine, BCH, and ADP. The antenna is necessary for GTP inhibition and ADP activation [31]. Similarly, EGCG does not inhibit the 'antenna-less' form of GDH, and is further evidence that EGCG is an allosteric inhibitor. Most importantly, EGCG inhibits HHS GDH mutants as effectively as wild type [84], making it a possible therapeutic lead compound.

The next step was to ascertain whether EGCG was active in tissue. Studies have demonstrated that GDH plays a major role in leucine stimulated insulin secretion (LSIS) by controlling glutaminolysis [49, 64]. Therefore, EGCG was tested on pancreatic β-cells using the perifusion assay [84]. Importantly, EGCG, but not EGC, blocked the GDH-mediated stimulation of insulin secretion by the β-cells but did not have any effect on insulin secretion, glucose oxidation, or cellular respiration during glucose stimulation where GDH is known to not play a major role in the regulation of insulin secretion. Therefore, EGCG is indeed a specific inhibitor of GDH both In-vitro and in-situ.

As shown in Fig. 7, EGCG inhibition of GDH-mediated insulin secretion in β-cells also extends to transgenic (TG) mouse tissue that expressed a gain of function of human GDH mutation, H454Y, in β-cells. As expected, the glutaminase inhibitor, DON, and EGCG are both able to block the HHS hyper-response to the addition of Gln. However, only EGCG was able to bring down the basal level of insulin release to that of WT tissue. As shown here, EGCG does not decrease insulin levels in WT tissue. This is likely due to GDH being kept mostly in a tonic state in the pancreas and its allosteric inhibition is only alleviated when the energy state of the mitochondria is low. Indeed, the ADP/EGCG antagonism may allow for an allosteric 'release valve' whereby even EGCG inhibition is abrogated by ADP when the need for amino acid catabolism is strong enough. This model is further supported by the amino acid metabolism studies that measure amino acids levels in the pancreatic tissue [85]. Under glucose rich conditions, there is no significant effect of EGCG on Glu/Gln levels in either TG or WT cells. This is likely due to nearly quiescent GDH activity because of the elevated levels of GTP and ATP. However, when Gln is the major carbon source, EGCG significantly blocks Glu metabolism in TG tissue while not having significant effects on WT tissue. Under such conditions, the GDH activity is expected to increase to respond to the energy needs of the mitochondria. Since the GDH activity is much higher in TG tissue, it follows that it will be more sensitive to EGCG inhibition.

While EGCG is a natural product with extremely low toxicity issues, it has several problems as a therapeutic agent [86]. It is poorly absorbed in the intestinal tract, is rapidly modified by enzymes such as catechol-O-methyltransferease (COMT), and its anti-oxidant activity makes it relatively unstable in solution. To validate our findings with EGCG, the more stable GDH inhibitor, hexachlorophene (HCP), identified in our previous high throughput screening (HTS) studies [39], was also examined. Exactly as was found with EGCG, HCP was very effective at blocking the hyper-response to Gln in TG tissue. However, likely because of its greater stability and hydrophobicity, the approximate EC_{50} for HCP in tissue is nearly the same as was found In-vitro with purified GDH [39]. This demonstrates that developing an effective therapeutic agent will require a balance between stability, toxicity, and bioavailability.

The remaining question was whether either of these lead compounds could control the HHS symptoms in the TG mice when administered orally. Due to its low toxicity, EGCG was selected for in-vivo application. An optimal drug for HHS should be able to block the hyperinsulinism

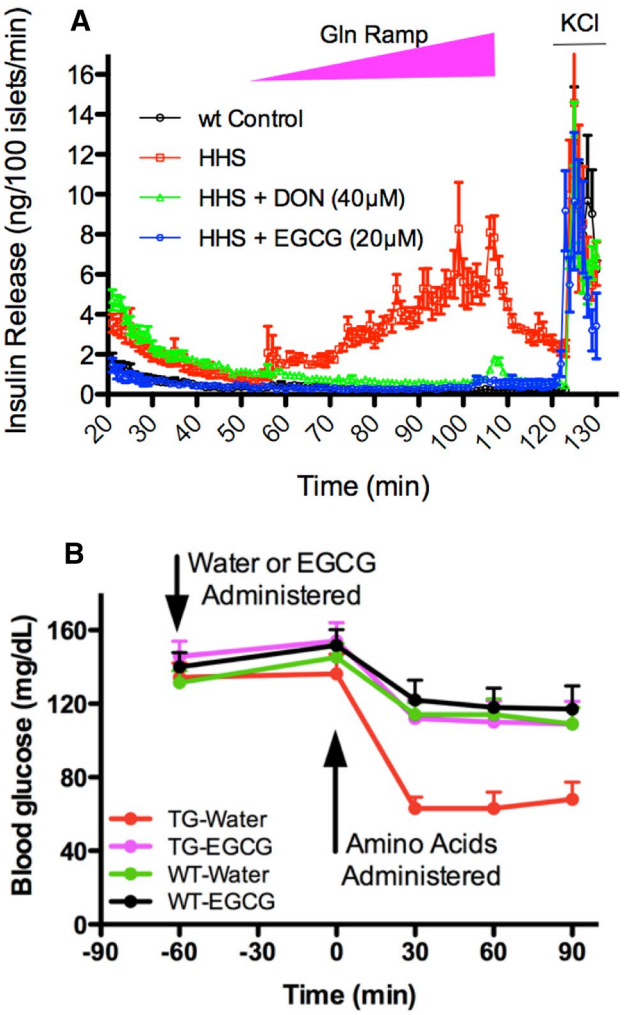

Fig. 7 The effect of EGCG on insulin secretion in H454Y transgenic mice β-islets and on the whole animal (Figures adapted from [85]). **a** TG tissue secretes insulin in response to a Gln ramp stimulation. This is not observed in WT islets, and glutamine-stimulated insulin secretion in TG islets is blocked by the glutaminase inhibitor, DON, and by the GDH inhibitor, EGCG. Note that EGCG, but not DON, brings the basal insulin secretion levels (T = 20 min) down to that of WT (data are mean ± S.E. (error bars), n = 3 for each group). The black line representing WT tissue maybe difficult to see because it lies directly under the TG+EGCG line (green). **b** This figure shows the effects of oral administration of EGCG on the hypersecretion of insulin in HHS transgenic mice. Plasma glucose levels in WT mice (n = 12 for water- or EGCG-treated mice) are essentially unaffected by oral administration of water or EGCG prior to the administration of the amino acid mixture. However, the plasma glucose levels rapidly drop in the HHS TG mice (n = 12) upon the administration of the amino acid mixture, but this is blocked when the animals are fed EGCG (n = 16) prior to the amino acid challenge. (Color figure online)

response upon the consumption of amino acids as well as elevate basal serum glucose levels. As shown in Fig. 7b, when EGCG is orally administered before challenging the TG mice with an amino acid mixture, the GDH-mediated hyperinsulinism is blocked [85]. In addition, as was first observed in the islet perifusion assays (Fig. 7A), chronic administration of EGCG during fasting improved the basal plasma glucose levels in the TG mice. Together, these results clearly demonstrate that it is possible to directly target the dysregulated form of GDH in HHS in-vivo. It remains to be seen whether such compounds can also alleviate serum ammonium levels and prevent the CNS pathology caused by HHS.

It is important to note that allosteric GDH inhibitors may have more applications than just treating HHS. Recent studies confirmed our observation that EGCG inhibits GDH in-situ and may be useful in treating glioblastoma [68]. In this work, EGCG was found to sensitize glioblastoma cells to glucose withdrawal and to inhibitors of Akt signaling and glycolysis. Subsequently, others demonstrated EGCG inhibition of GDH activity may be useful in treating the tuberous sclerosis complex (TSC) disorder [87]. Nearly all of the TSC1/2 -/- cells that were deprived of glucose and given rapamycin died upon administration of EGCG. As expected, EGCG effects were reversed if GDH mediated oxidation of glutamate was circumvented by the addition of α-KG, pyruvate, or aminooxyacetate. Not only do these studies validate our findings, but also demonstrate that a non-toxic GDH inhibitor could be a synergistic tool in treating tumors.

GDH as a Possible Target for Diabetes Type II Treatment

From the results reviewed above, it is clear that activation of GDH activity can stimulate insulin secretion. It naturally follows that GDH might be a possible target for type II diabetes treatment by the application of a pharmaceutical activator of the enzyme. Recently, this has been shown to be a possibility with the known activator, BCH [88]. Diabetic db/db (C57BLKS/J-leprdb/leprdb) mice were given oral doses of BCH for 6 weeks. It was found that BCH blocked the high glucose induced GSIS inhibition and the high glucose/palmitate induced reduction of insulin expression in INS-1 cells. Further, BCH reduced the apoptotic cell death of INS-1 cells during the high glucose/palmitate treatment. Together, BCH treatment improved glucose tolerance in the db/db mice possibly by enhancing insulin secretion as well protecting the β-cells and islet architecture.

The Search for New GDH Activators

While HHS is an extreme form of enhanced insulin secretion by GDH stimulation [62, 89], diabetes type II might be treated by a controlled and limited enhancement of GDH activity. Currently, the only synthetic GDH activator is BCH. BCH is a poor drug with an ED_{50} of ~ 10 mM [84],

a solubility of only 100 mM, and is not entirely specific for GDH (e.g. inhibits glutamine transport [90] and activates apoptosis in cancer cells [91]). The poor efficacy and off-site effects limits any in-vivo testing, and therefore the feasibility of treating diabetes II via GDH absolutely requires more potent compounds.

To search for possible activators, the previous HTS method [39, 76] was revisited with some changes in the assay protocol [92]. Firstly, rather than measuring the endpoint of the reaction after 30–60 min, the velocity was measured immediately after the addition of the enzyme. The second change in the protocol was the addition of GTP to identify new compounds that abrogate GTP inhibition. One of the best activators was identified as N1-[4-(2-aminopyrimidin-4-yl)phenyl]-3-(trifluoromethyl)benzene-1-sulfonamide, or compound 75-E10 in the Maybridge library (Fig. 8).

Very similar to ADP, 75-E10 has the greatest effect in the presence of GTP. In the absence of GTP, 75-E10 causes modest activation but completely removes GTP inhibition at 100 µM (Fig. 8b). In the presence or absence of GTP, the apparent activation constant for 75-E10 is ~53 µM. Therefore, the apparent binding constant for the drug is the same whether or not GTP is present. However, because 75-E10 removes GTP inhibition, the apparent maximum activation is markedly different in the presence of GTP. In the absence of GTP, the drug causes a maximum activation of ~100%. However, in the presence of 50 µM GTP, this is increased to ~337%. Therefore, in general, 75-E10 is remarkably similar in effects to ADP, leucine, and BCH where the majority of the activation is the abrogation of allosteric inhibitors rather than direct activation of the enzyme.

The next step was to determine where 75-E10 might be binding to cause these effects. Fortunately, 75-E10 fluoresces quite strongly and this could be used for binding analysis. When 75-E10 binds to GDH it undergoes a clear blue shift in emission maximum, as is typical when fluorophores move out of water and into a less polar environment. Using this blue shift in fluorescence upon binding, the direct binding of the drug to GDH was measured. The binding data agreed well with a single site binding equation and a Kd of ~22 µM. For comparison, the Kd for ADP to the GDH alone is ~3 µM but increases to 17 µM in the presence of NAD(P)H [10].

The blue shift in 75-E10 upon binding also allowed for an easy way to look at possible binding competition between the various allosteric ligands. While the addition of very high concentrations of GTP did not affect the spectrum of the bound 75-E10, the addition of the same amount of ADP causes a spectral shift back towards the free state of 75-E10. This could be due to a direct competition between 75-E10 and ADP or due to ADP binding to a separate site and allosterically blocking 75-E10 binding. Since it is apparent that ADP competes with the binding of the compound, an independent way to determine the binding constant is to measure the competition between ADP and 75-E10. The observed data fit very well to a simplified competition equation and yielded nearly the same Kd as when 75-E10 binding was measured alone. Albeit not proof, it lends circumstantial evidence that 75-E10 and ADP bind to the same site.

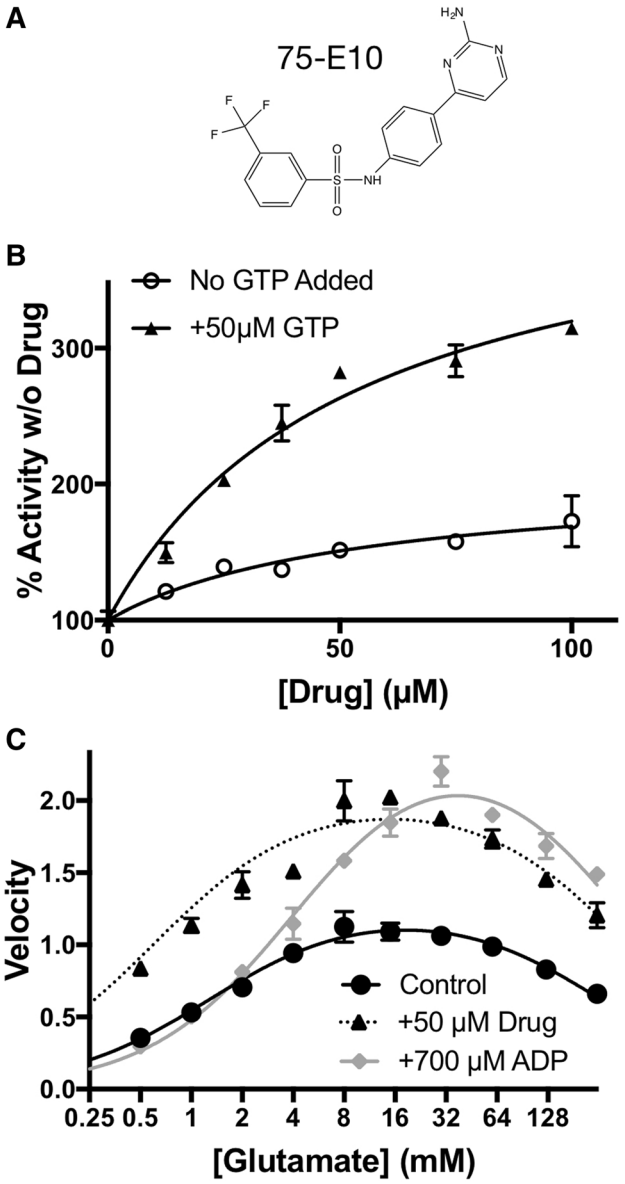

Fig. 8 Identification of a new GDH activator using high throughput screening. By screening the compound library with GTP in the assay, N1-[4-(2-aminopyrimidin-4-yl)phenyl]-3-(trifluoromethyl) benzene-1-sulfonamide, Maybridge Hitscreen compound 75-E10, was identified as a promising new activator of GDH (figures adapted from [92]) **a** The structure of 75-E10. **b** This figure shows the effects of 75-E10 on GDH in the presence and absence of GTP. As with leucine and ADP, 75-E10 has a small effect on GDH activity alone (~50% activation) but a much larger effect when abrogating GTP inhibition (~330% activation). **c** This graph shows that 75-E10 is more effective than the natural activator, ADP, with a > 10-fold better ED_{50} and activation of the enzyme over a broader range of conditions

These results are very encouraging since it is typically easier to identify inhibitors rather than activators in HTS. By choosing conditions for the screen that favor a highly-inhibited state of GDH, it was possible to identify 75-E10 with nearly 1000-fold higher efficacy than the only other synthetic activator of GDH, BCH, and more effective than the natural activator, ADP (Fig. 8c). The relative ease of finding such activators demonstrates how malleable GDH is with regard to allosteric regulation. This is likely due to all of the motions associated with catalytic turnover [65] and the inter-subunit communication afforded by the antenna [31], that create numerous places on the enzyme that either facilitate or inhibit the opening and closing of the catalytic cleft.

Conclusions

There is growing interest in GDH since it is now clear that its role in animals is multifaceted. It is clear that the enzyme is not working at equilibrium and that it is operating in the oxidative deamination direction. GDH has evolved through the epochs to play a major role in insulin homeostasis, regulation of several CNS processes, cell growth, and ureagenesis. The enzyme is able to play such a key role in so many processes because the complicated balance of so many different regulators, many of which act antagonistically or agonistically with each other. The complex motions that the enzyme undergoes during each catalytic cycle creates a number of pockets in the protein that are utilized to allosterically regulate activity by both metabolites and other mitochondrial enzymes. Further, these regulators interact with each other such that GDH activity is not simply turned 'off' or 'on' but rather finely tuned depending upon the balance of numerous metabolites from glycolysis and fatty acid catabolism. Work by several groups have now shown that inhibitors to GDH might not only be useful for controlling HHS, but also have potential in affecting tumor growth. While In-vitro studies with the synthetic leucine analog, BCH, have suggested that GDH activators could be used to stimulate insulin secretion, further work is needed to identify more efficacious compounds for an in-vivo proof of concept. Work has only just begun to understand what role GDH plays in CNS development and regulation. It seems, therefore, that animals took an ancient enzyme with a rather simple catabolic function and evolved onto it many layers of allosteric regulation so that it can affect such varied processes in disparate tissues.

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