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Glutamate Dehydrogenase: Structure, Allosteric Regulation, and Role in Insulin Homeostasis

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Abstract

Glutamate dehydrogenase (GDH) is a homohexameric enzyme that catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate. Only in the animal kingdom is this enzyme heavily allosterically regulated by a wide array of metabolites. The major activators are ADP and leucine and inhibitors include GTP, palmitoyl CoA, and ATP. Spontaneous mutations in the GTP inhibitory site that lead to the hyperinsulinism/hyperammonemia (HHS) syndrome have shed light as to why mammalian GDH is so tightly regulated. Patients with HHS exhibit hypersecretion of insulin upon consumption of protein and concomitantly extremely high levels of ammonium in the serum. The atomic structures of four new inhibitors complexed with GDH complexes have identified three different allosteric binding sites. Using a transgenic mouse model expressing the human HHS form of GDH, at least three of these compounds blocked the dysregulated form of GDH in pancreatic tissue. EGCG from green tea prevented the hyper-response to amino acids in whole animals and improved basal serum glucose levels. The atomic structure of the ECG–GDH complex and mutagenesis studies is directing structure-based drug design using these polyphenols as a base scaffold. In addition, all of these allosteric inhibitors are elucidating the atomic mechanisms of allostery in this complex enzyme.

Keywords

Glutamate dehydrogenase; Insulin regulation; Polyphenols; Allostery; Subunit communication; Protein dynamics

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Glutamate dehydrogenase (GDH) is found in all organisms and catalyzes the reversible oxidative deamination of L-glutamate to 2-oxoglutarate using NAD^+ and/or $NADP^+$ as coenzyme [1]. In nearly all organisms, GDH is a homohexameric enzyme composed of subunits comprised of ~500 residues in animals and ~450 residues in the other kingdoms. While the chemical details of the enzymatic reaction have been tightly conserved through the epochs, the metabolic role of the enzyme has not. Most striking is the fact that GDH from animal sources is allosterically regulated by a wide array of metabolites while it is mainly regulated at the transcriptional level in the other kingdoms.

In animals, the two major opposing allosteric regulators, ADP and GTP, appear to exert their effects via abortive complexes [2–4]. Abortive complexes are formed when the product is replaced by substrate before the reacted coenzyme has a chance to dissociate; GDH·glutamate·NAD(P)H in the oxidative deamination reaction and GDH·2-oxoglutarate·$NAD(P)^+$ in the reductive amination reaction. Once these complexes form, coenzyme binds very tightly and slows down enzymatic turnover by inhibiting product release. ADP is an activator believed to act, at least in part, by destabilizing the abortive complex [2, 3]. In contrast, GTP is a potent inhibitor and is thought to act by stabilizing abortive complexes [5]. GTP binding is antagonized by phosphate [6] and ADP [7], but is proposed to be synergistic with NADH bound in the non-catalytic site [6]. Finally, ADP and GTP bind in an antagonistic manner [7] either due to steric competition or due to competing effects on abortive complex formation. As discussed below, it is likely that these regulators act by modulating the enzyme dynamics [8, 9].

Mammalian GDH is also regulated by several other types of metabolites. Leucine, as well as some other monocarboxylic acids, has been shown to activate mammalian GDH [10] by increasing the rate-limiting step of coenzyme release in a manner similar to ADP [11]. Since leucine is a weak substrate for GDH, one binding site is the active site, but it is unclear whether there is a second, allosteric site. Palmitoyl-CoA [12] and diethylstilbestrol [13] are inhibitors of mammalian GDH, but nothing is known about their binding locations.

The Structure of Animal GDH

The structure of GDH (Fig. 1) is essentially two trimers of subunits stacked directly on top of each other with each subunit being composed of at least three domains [8, 9, 14, 15]. The bottom domain makes extensive contacts with adjacent subunits from the other trimer. 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 vast majority of protists. The antenna from each subunit lies immediately behind the adjacent, counter-clockwise neighbor within the trimer. Since these intertwined antennae are only found in the forms of GDH that are allosterically regulated by numerous ligands, it is reasonable to speculate that it plays an important role in regulation.

GDH Dynamics

From the structures of GDH with and without active site ligands, it is possible to observe the closure of the active site cleft and the associated conformational changes that occur throughout the hexamer during each catalytic cycle [8, 9, 14, 15]. When substrate is released from the deep recesses of the active site cleft, the entire enzyme undergoes a number of large conformational changes. 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 is translated up through the antenna and gives it a clockwise motion. The short distended helix in the descending loop of the antenna coils back into a longer, better ordered helix 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 a rigid units away from each other, opening the cavity at the core of the hexamer. As is further detailed in subsequent sections, it is apparent that animal GDH is controlled allosterically via ligand interactions at a number of these flex points.

The GTP Inhibition Site

GTP is a potent allosteric inhibitor for the reaction and binds at the base of the antenna, wedged in between the NAD binding domain and the pivot helix [14, 15] (Fig. 2). 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 and concomitantly increasing the binding affinity of substrate and coenzyme [5, 6, 16]. 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. This explains why, in terms of inhibition, GTP ≫ GDP > GMP [2]. Therefore, when the mitochondrial energy level is high, GTP (and ATP) levels will be elevated and 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 that is also a second NAD(H) binding site (Fig. 3). The existence of a second NADH binding site per subunit was demonstrated both kinetically and by binding analysis [17–19]. 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 [19]. Similarly, GTP also increases the affinity and binding stoichiometry [6]. This second coenzyme site strongly favors NADH over NADPH with Kd’s of 57 μM 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 [9, 15], this was first suggested by ADP binding competition with NAD^+ [20] and NADH [7]. Further, 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. Since, in general terms, NADPH is involved in anabolic reactions in the cell while NADH is important for catabolic processes, it is possible that this regulation offers a feedback mechanism to curtail glutamate oxidation when catabolic reductive potentials (NADH) are high.

ADP Activation

In nearly every way, ADP acts in a manner opposite to NADH binding to this site. In the oxidative deamination reaction, ADP activates at high pH, but inhibits at low pH with either NAD^+ or $NADP^+$ as coenzyme [4]. 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 [4]. 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 (2-oxoglutarate) 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].

ADP and the second molecule of NAD(H) binds behind the NAD binding domain and immediately under the pivot helix (Fig. 3a) [9]. As shown in Fig. 3b, R463 lies on the pivot helix and interacts with the phosphates of the bound ADP. For this review, all residue numbers correspond to human GDH. It was proposed that this interaction might facilitate the rotation of the NAD binding domain and the release of product [9]. To test this, R463 was mutated to an alanine and this led to a loss in ADP activation. This essentially suggests that ADP activates the reaction by ‘pulling’ on the back of the NAD binding domain to help open the active site cleft and facilitating product release. More precisely, ADP likely decreases the energy required to open the catalytic cleft.

The physiological role of ADP activation is easily understood; when the energy level of the mitochondria is low and ADP levels are high, the catabolism of glutamate is accelerated for energy production. However, the possible in vivo role of NADH inhibition is less clear. Since NADH inhibition is observed at concentrations above 0.2 mM (e.g. see [21]), but only reaches ~50 % inhibition at 1 mM NADH, NADH inhibition seems more likely to work synergistically with GTP regulation; under conditions of high reductive potential, NADH acts with GTP to keep GDH in a tonic state.

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 [11]. 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.

Role of Allosteric Regulation in Insulin Homeostasis

The difference between the allosteric regulation of GDH from animals and the other kingdoms has been known for decades, but possible roles for allosteric regulation in mammalian GDH is only starting to emerge. Of growing interest is the fact that loss of allosteric inhibition of GDH causes inappropriate insulin secretion. There have been three GDH-mediated forms of hyperinsulinism identified thus far: hyperinsulinism/hyperammonemia (HHS) due to mutations that abrogate GTP inhibition, mutations in NAD-dependent deacetylase (SIRT4), and knock out mutations of short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD).

HHS

HHS was one of the first diseases that clearly linked GDH regulation to insulin and ammonia homeostasis [22]. In brief, the mitochondrion of the pancreatic β-cells plays an integrative role in the fuel stimulation of insulin secretion. The current concept is that mitochondrial oxidation of substrates increases the cellular phosphate potential that is manifested by a rise in the $ATP^{4-}/MgADP^{2-}$ ratio. The elevated ATP concentration closes the plasma membrane K_{ATP} channels, resulting in the depolarization of the membrane potential. This voltage change across the membrane opens voltage gated Ca^{2+} channels. The rise of free cytoplasmic Ca^{2+} then leads to insulin granule exocytosis.

The connection between GDH and insulin regulation was initially established using a non-metabolizable analog of leucine [23, 24], BCH (β-2-aminobicycle(2.2.1)-heptane-2-carboxylic acid). These studies demonstrated that activation of GDH was tightly correlated with increased glutaminolysis and release of insulin. In addition, it has also been noted that factors that regulate GDH also affect insulin secretion [25]. Subsequently, it was postulated that glutamine could also play a secondary messenger role and that GDH plays a role in its regulation [26–28]. The in vivo importance of GDH in glucose homeostasis was demonstrated by the discovery that a genetic hypoglycemic disorder, the HHS syndrome, is caused by loss of GTP regulation of GDH [22, 29, 30]. Children with HHS have increased β-cell responsiveness to leucine and susceptibility to hypoglycemia following high protein meals [31]. This is likely due to uncontrolled catabolism of amino acids yielding high ATP levels that stimulate insulin secretion as well as high serum ammonium levels. The elevation of serum ammonia levels reflects the consequence of altered regulation of GDH leading to

increased ammonia production from glutamate oxidation, and possibly also impaired urea synthesis by carbamoylphosphate synthetase due to reduced formation of its activator, *N*-acetyl-glutamate, from glutamate. Essentially, this genetic lesion disrupts the regulatory linkage between glucose oxidation and amino acid catabolism. 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 [26, 27].

While the glucose and ammonium levels in patients with HHS are alone sufficient to potentially cause damage to the central nervous system (CNS), recent studies have suggested a high correlation between HHS and childhood-onset epilepsy, learning disabilities, and seizures [32]. Some of these pathologies have been shown to be unrelated to serum glucose and ammonium levels. This is not entirely surprising considering the importance of glutamate and its derivative, γ-aminobutyric acid, as neurotransmitters. The current treatment for HHS is to pharmaceutically control insulin secretion (e.g. diazoxide, a potassium channel activator) but this does not address the serum ammonium and CNS pathologies.

SIRT4 Mutations

Sirt2 or sirtuins (silent mating type information regulation 2 homolog) are found in all organisms and most are NAD-dependent protein deacetylases. The sirtuins have been shown to be implicated in aging and regulate transcription, involved in stress response, and apoptosis. Studies have shown that SIRT4, a mitochondrial enzyme, uses NAD to ADP-ribosylate GDH and inhibit its activity [33, 34]. When SIRT4 knockout mice were generated, the loss of SIRT4 activity led to the activation of GDH and, much like HHS, upregulated amino acid-stimulated insulin secretion [34]. In addition, they found that, with regard to that ADP-ribosylation, GDH activity in SIRT4 −/− mice was similar to mice on a calorie restriction diet regime. This suggests that normally SIRT4 in the β cell mitochondria represses GDH activity by ADP-ribosylation and this regulation is removed during times of low caloric intake.

SCHAD Mutations

A form of recessively inherited hyperinsulinism has been identified to be associated with a deficiency of a mitochondrial fatty acid β-oxidation enzyme, the SCHAD encoded by the *HADH* gene on 4q [35–37]. Children with this defect have recurrent episodes of protein-induced hypoglycemia [38] that can be controlled with the K_{ATP} channel agonist diazoxide. These patients also have serum accumulation of fatty acid metabolites such as 3-hydroxybutyryl-carnitine and urinary 3-hydroxyglutaric acid [35, 37]. Similar to HHS, these patients also have severe dietary protein sensitivity [35]. However, the loss of SCHAD activity is at odds with increased insulin secretion observed in HHS since it is expected to decrease ATP production. In addition, other genetic disorders of mitochondrial fatty acid oxidation do not lead to hyperinsulinism [39].

Recent studies have now explained this seeming paradox by suggesting that SCHAD regulates GDH activity via protein–protein interactions [40]. From immunoprecipitation and pull-down analysis, it is apparent that GDH and SCHAD interact as had been previously suggested [41]. This interaction inhibits GDH activity by decreasing GDH affinity for substrate. This effect was found to be limited to the pancreas presumably because of the relatively high levels of SCHAD found in this tissue. This would also explain why SCHAD deficient patients do not have a concomitant increase in serum ammonium levels as observed in HHS. These results are particularly interesting since it strongly suggests that part of GDH regulation comes from associating as a large multienzyme complex in the mitochondria. This also suggests a linkage between fatty acid and amino acid oxidation and fits in well with the evolution of allostery as discussed in the next section.

Evolution of GDH Allostery

It has been known for some time that animal GDH contains an internal 48-residue insert compared to GDH from the other kingdoms. From the structure of animal GDH, we now know this insert forms the antenna region (Fig. 1). What was not at all clear was when and why the antenna evolved and whether it was linked to the complex pattern of allostery in animal GDH. The only organisms other than animals that have this 48-residue insert are the *Ciliates*. This possible evolutionary ‘missing link’ was then further analyzed via kinetic and mutagenesis analyses using tetrahymena GDH (tGDH) [42]. Like mammalian GDH, tGDH is activated by ADP and inhibited by palmatoyl CoA. However, like bacterial GDH, tGDH is coenzyme specific (NAD(H)) and is not regulated by GTP or leucine. Therefore, with regard to allosteric regulation, tGDH is indeed an evolutionary ‘missing link’ between bacterial and animal GDH.

From structural studies, it was clear that the antenna was not directly involved in ADP or GTP binding [9, 14, 15]. However, when the antenna on human GDH was genetically replaced by the short loop found in bacterial GDH, the enzyme lost GTP, ADP, and Palmitoyl-CoA regulation [42]. When the *Ciliate* antenna was spliced onto human GDH, this hybrid enzyme had a fully functional repertoire of mammalian allostery. This demonstrated that the antenna in *Ciliate* GDH is capable of transmitting the GTP inhibitory signal, but GTP regulation was apparently not needed in the *Ciliates*.

This suggests that GDH allostery evolved in at least a two-step process. The first evolutionary step may have been due to the changing functions of the cellular organelles as the *Ciliates* branched off from the other kingdoms. In the other eukaryotic organisms, all fatty acid oxidation occurs in the peroxisomes [43, 44]. In the *Ciliates*, fatty acid oxidation is shared between the peroxisomes and the mitochondria [45, 46]. Eventually, all medium and long chain fatty oxidation moved into the mitochondria in animals [47, 48]. This pattern of regulation suggests that the catabolism of amino acids is down regulated when there are sufficient levels of fatty acids. Only when the mitochondria have run low of fatty acids and its energy state is low (i.e. high ADP levels) will amino acids be catabolized. Therefore, allostery evolved to coordinate the growing complexity of mitochondrial metabolism and the antenna feature is needed to exact this regulation.

The second step of evolution was to add leucine and GTP allostery as animals needed even more sophisticated and rapid-response regulation of GDH activity. In animals, GDH is found in high levels in the CNS, pancreas, liver, and kidneys. In each organ, the levels of glutamate need to be controlled by very different physiological signals. These disparate roles for GDH are probably why GDH in animals has far more allosteric regulation than all other organisms.

In the pancreas, GDH needs to be activated when amino acids (protein) are ingested to promote insulin secretion and appropriate anabolic effects on peripheral tissues. In the glucose-fed state, triphosphate levels are high and GDH needs to be inhibited to redirect amino acids into glutamine synthesis in order to amplify insulin release. In the liver, GDH needs to be suppressed when other fuels, such as fatty acids, are available, but increased when surplus amino acids need to be oxidized. To this end, mammals added layers of regulation onto *Ciliate* GDH to include leucine activation and GTP inhibition. The choice of leucine as a regulator is likely not an accident, because leucine is an essential amino acid not made by humans and is also one of the most abundant amino acids in protein, making it a good measure of protein consumption. Similarly, the marked sensitivity of GDH for GTP over ATP is also not likely accidental. 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 there is a slow mitochondria/cytoplasm exchange rate. Therefore, the GTP/GDP ratio is much better metric of TCA cycle activity than the ATP/ADP ratio. Indeed, it has been demonstrated that mitochondrial GTP, but not ATP, regulates glucose-stimulated insulin secretion [49]. This is also 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 GTP, and more insulin will be released. Therefore, the addition of GTP and leucine regulation to GDH makes it acutely sensitive to glucose and amino acid catabolism with obvious implications for insulin homeostasis. It is also likely that this complex network of allostery was needed to accommodate the differing regulation needed by the CNS and ureagenesis.

High Throughput Screening for New Allosteric Inhibitors

While the hypersecretion of insulin can be controlled with compounds such as diazoxide [50], this does not address the serum ammonium and CNS pathologies. To this end, high throughput screening was used to find new inhibitors of GDH to control the dysregulated GDH in HHS [51] and some of the compounds are shown in Fig. 4. From the highly disparate chemical properties of the lead compounds, it seemed more than likely that the various classes were binding to different allosteric sites on GDH. The atomic structures of the various GDH-drug complexes not only offer a means to elucidate how these new allosteric sites can inhibit GDH activity, but are also invaluable for further drug design.

The Structure of Hexachlorophene (HCP) Bound to GDH

In the GDH–HCP complex [52], six molecules of HCP form a ring in the inner cavity of the hexamer (Fig. 5b, d). The majority of the interactions between HCP and GDH are hydrophobic, and there is a ring of aromatic stacking interactions. HCP binds in two orientations. In the first, the rings of HCP approximately stack against two Y190 side-chains 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 to 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 do not bind to the same site as HCP (Fig. 5b, c) [52]. While HCP binds to the inner core, these two drugs bind halfway between the core and the exterior of the hexamer. The binding environments of the two drugs are essentially identical. 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 drugs, therefore, appear to directly bind to the area that compresses during mouth closure.

Some very recent structural results on GDH from an extremely thermophilic bacteria, *Thermus thermophilus*, suggest that this bithionol/GW5074 binding site might be involved in leucine allosteric activation [53]. There are two forms of GDH in *T. thermophilus*, GdhA and GdhB, that can form various combinations of GdhA/GdhB heterohexamers. GdhA is catalytically inactive and may serve a regulatory function while GdhB catalyzes the reaction. Unlike GDH from non-animal sources, *T. thermophilus* GDH is activated by leucine. This is particularly unusual since even GDH from Tetrahymena, that has an antenna, is not regulated by leucine [42]. From the structures of GdhB complexed with glutamate and $GdhA_2/GdhB_4$ complexed with leucine, Tomita, et al. [53], found that these amino acids also bind at the subunit interface immediately adjacent to the bithionol/GW5074 site. The location of this possible allosteric leucine activation site was supported by mutations of the contact residues that abrogated leucine activation. The connection between this site and leucine activation in animal GDH needs to be confirmed, but it is not at all clear why *T. thermophilus* would need leucine regulation while other bacteria does not.

Structure of ECG Bound to GDH

This binding site for EGCG/ECG is quite different than the previous three compounds (Fig. 6). These polyphenols are extremely hydrophilic and ECG interactions with GDH are dominated by polar interactions and bind to ADP site [54]. 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 [54]. This is the same as what was observed when crystallizing the ADP/GDH complex [9].

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). From this we suggested that ADP might be facilitating enzymatic turnover by decreasing the energy required to open the catalytic cleft [9]. Mutagenesis studies on the ECG/EGCG binding site suggest it may be more complicated than that (Fig. 7) [54]. The guanidinium group of R90 stacks up against the aromatic rings in both ECG [54] and ADP [9] (Figs. 3b, 6b). 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 though R90 is quite distal to the GTP site (Fig. 7c). 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 (Fig. 6b). 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 (Fig. 7a, b). 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 (Figs. 3b, 6b). 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 as the R463A mutation that eliminates ADP activation by eliminating the charge interaction between R463 and ADP [9]. S397 lies at the base of the antenna (Fig. 6b) and the S397I mutation greatly destabilizes the enzyme while abrogating both GTP and ADP regulation (Figs. 7c, d). 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 [42].

Possible Therapeutics for GDH-Mediated Insulin 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 [54]. Since EC or EGC were not active against GDH (Fig. 4), but have the same anti-oxidant activity as ECG and EGCG, the anti-oxidant property of these catechins cannot be relevant to GDH inhibition [55]. 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} [55]. EGCG inhibition is non-competitive and, similar to GTP inhibition, is abrogated by leucine, BCH, and ADP. As noted above, the antenna is necessary for GTP inhibition and ADP activation [42]. 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 [55], 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 by

controlling glutaminolysis [26, 27]. Therefore, EGCG was tested on pancreatic β-cells using the perifusion assay [55]. 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. 8a, 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 [54]. 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 [56]. It is poorly absorbed in the intestinal tract, is rapidly modified by enzymes such as catechol-*O*-methyltransferease, and its anti-oxidant activity makes it relatively unstable in solution. To validate our findings with EGCG, the more stable GDH inhibitor, HCP, identified in our previous HTS studies [51], was also examined (Fig. 8b). 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 [51]. 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 response upon the consumption of amino acids as well as elevate basal serum glucose levels. As shown in Fig. 9a, when EGCG is orally administered before challenging the TG mice with an amino acid mixture, the GDH-mediated hyperinsulinism is blocked [54]. In addition, as was first observed in the islet perifusion assays (Fig. 8a), chronic administration of EGCG during fasting improved the basal plasma glucose levels in the TG mice (Fig. 9b). 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 elevate 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 [57]. 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 [58]. 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 2-oxoglutarate, 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 an pharmaceutical activator of the enzyme. Recently, this has been shown to be a possibility with the known activator, BCH [59]. 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.

Conclusions

GDH may have initially evolved these large conformational changes to improve catalytic efficiency [60]. This complex ballet of motion creates numerous sites on the protein where ligands can bind and modulate catalytic activity. Through this allosteric regulation, new functions and roles for GDH were created for GDH rather than needing to create entirely new gene products to accommodate the changing needs of the cell. While it seems that GDH regulation is overly complex for an enzyme involved in such a mundane chemical reaction, it is in fact remarkable that a single enzyme can control levels of glutamate in the CNS, ureagenesis in the liver, and insulin secretion in the pancreas. The findings that compounds can effectively up and down regulate GDH activity in vivo hold promise that it may be possible to both control HHS and affect diabetes.

Acknowledgments

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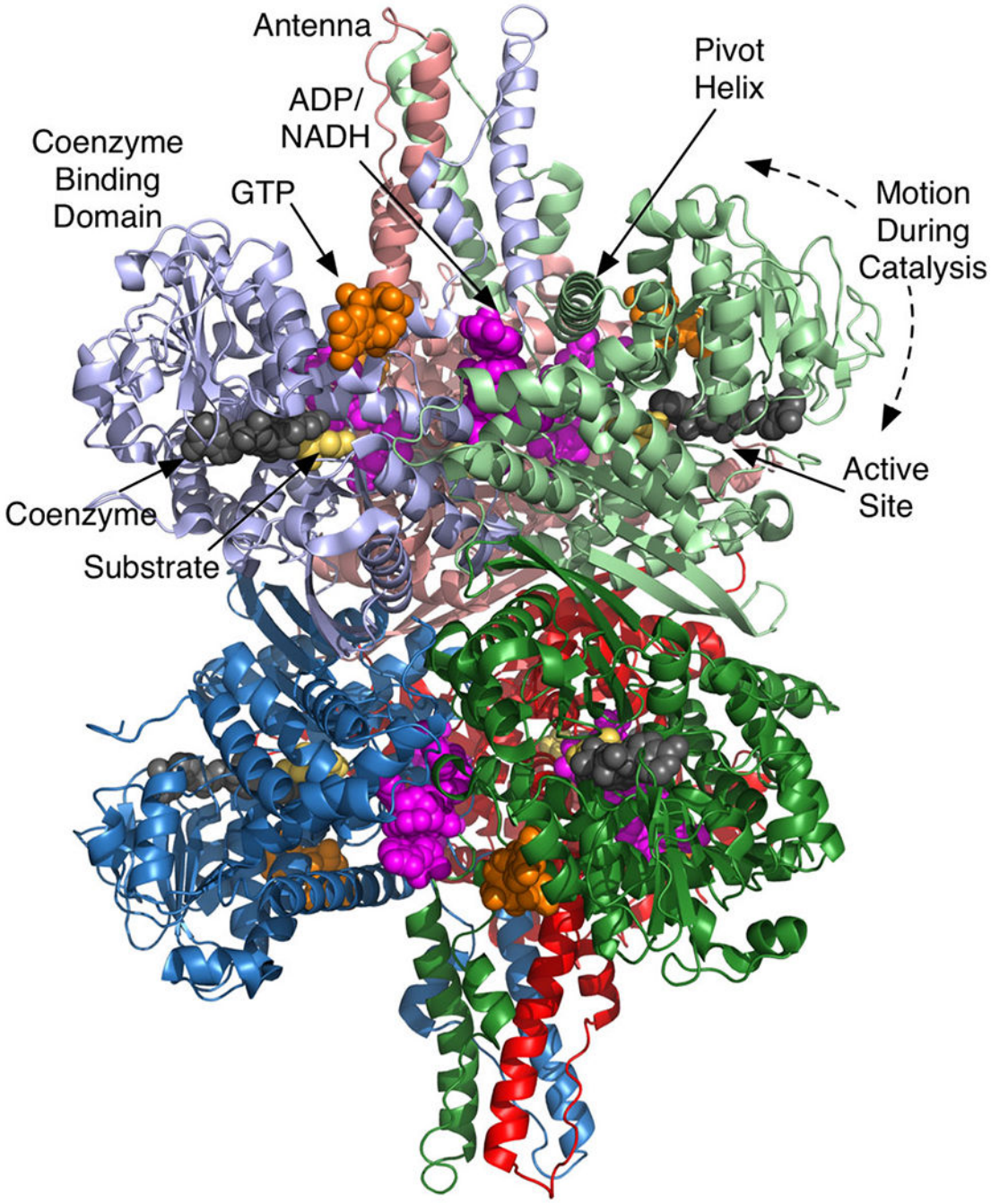

Fig. 1.

Structure of animal GDH. Each subunit is represented by a ribbon diagram and in different colors. The *arrow* highlights the key movement of the NAD binding domain during catalysis. The bound GTP, NAD(P)(H), glutamate, and ADP ligands are highlighted in *orange, grey, yellow* and *mauve*

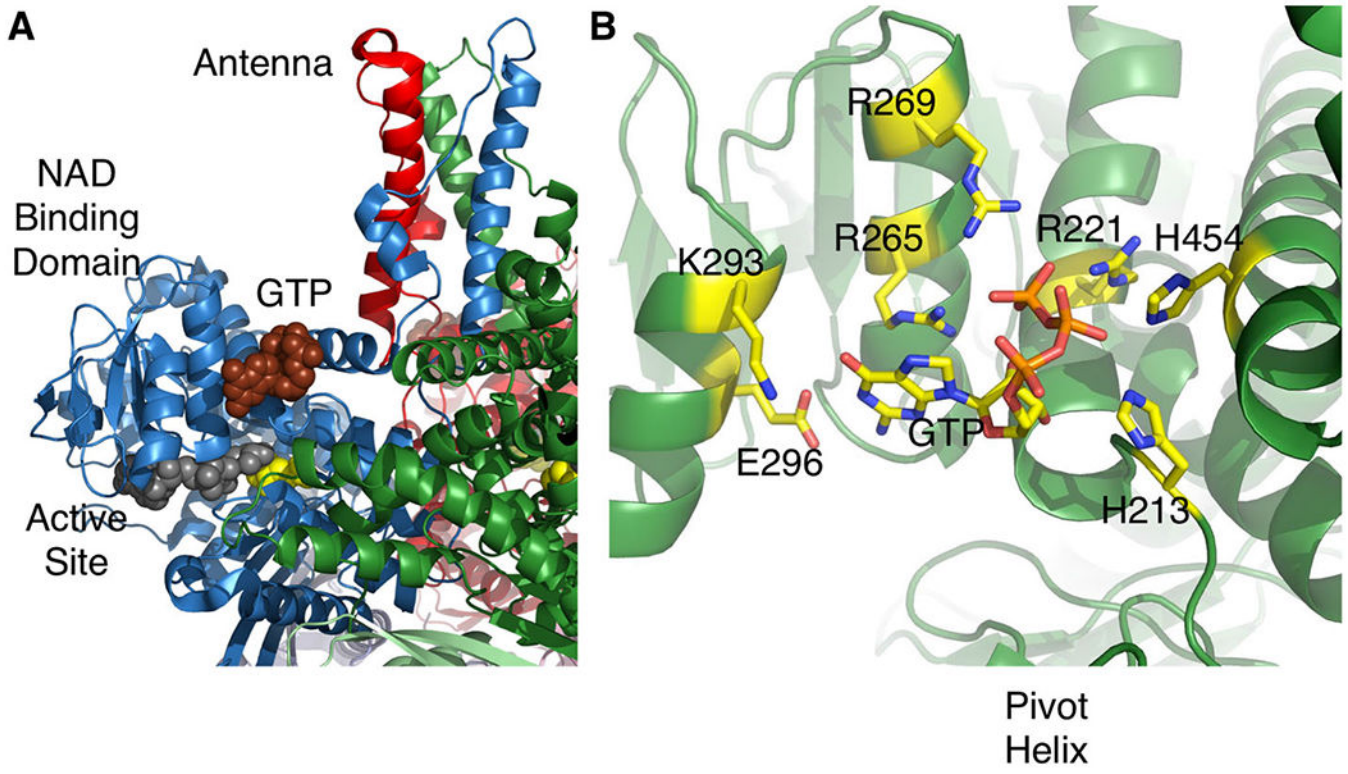

Fig. 2.

Location of the inhibitor, GTP, binding site [14]. All residue numbers for all figures correspond to human GDH. **a** The structure of GTP (*brown spheres*) bound to the closed conformation of GDH. **b** Magnification of GTP bound to GDH represented by stick figures. Some of the key ligating protein residues are also highlighted

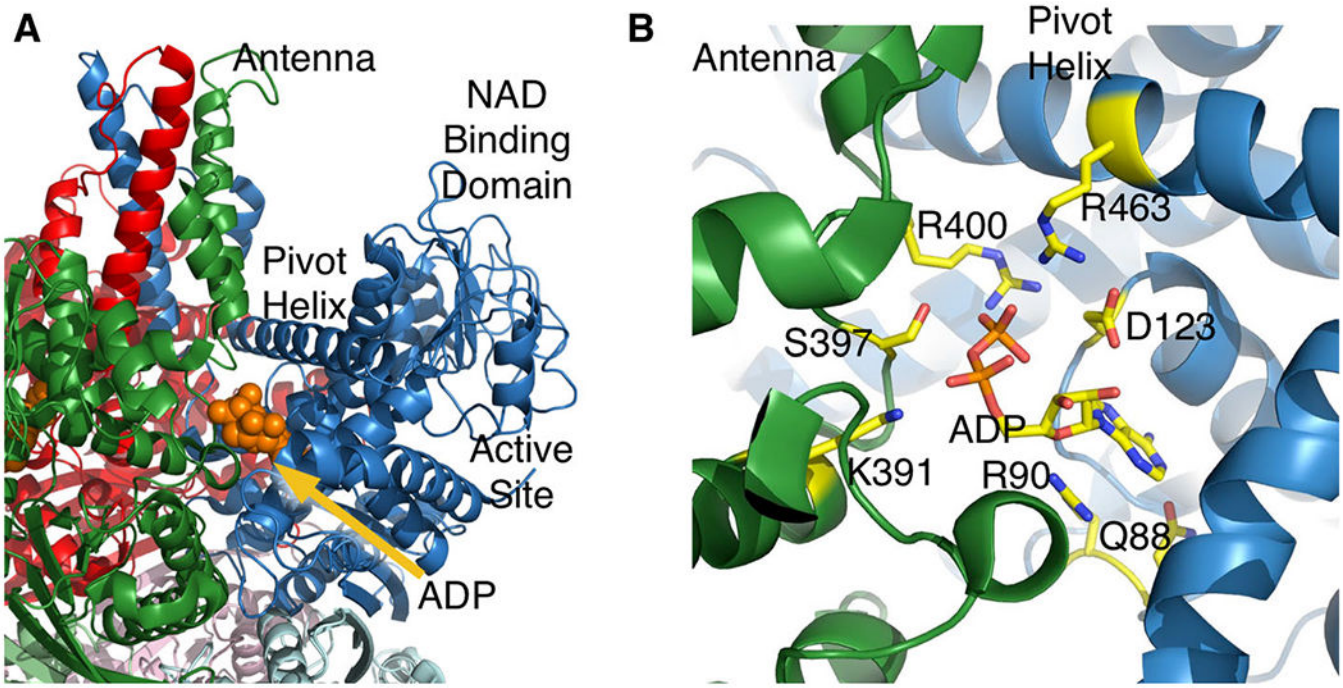

Fig. 3.

Location of the activator, ADP, binding site [9]. **a** ADP (*orange spheres*) binds behind the catalytic cleft, under the *pivot helix*. **b** Magnification of the bound ADP molecule with some of the key binding residues highlighted as stick figures

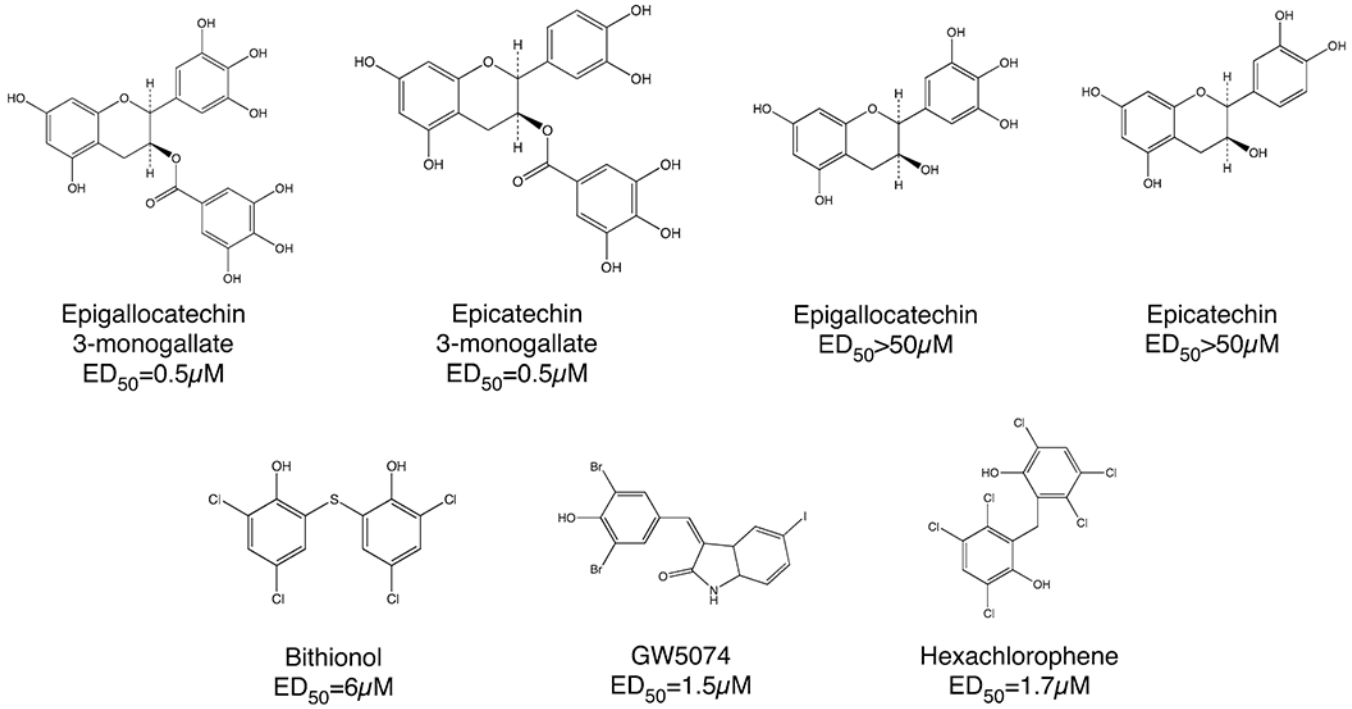

Fig. 4.

The structures of some of the bioactive compounds found from the high throughput screening [51]. Note that epigallocatechin and epicatechin are inactive but shown for comparison. Also noted are the approximate ED50's

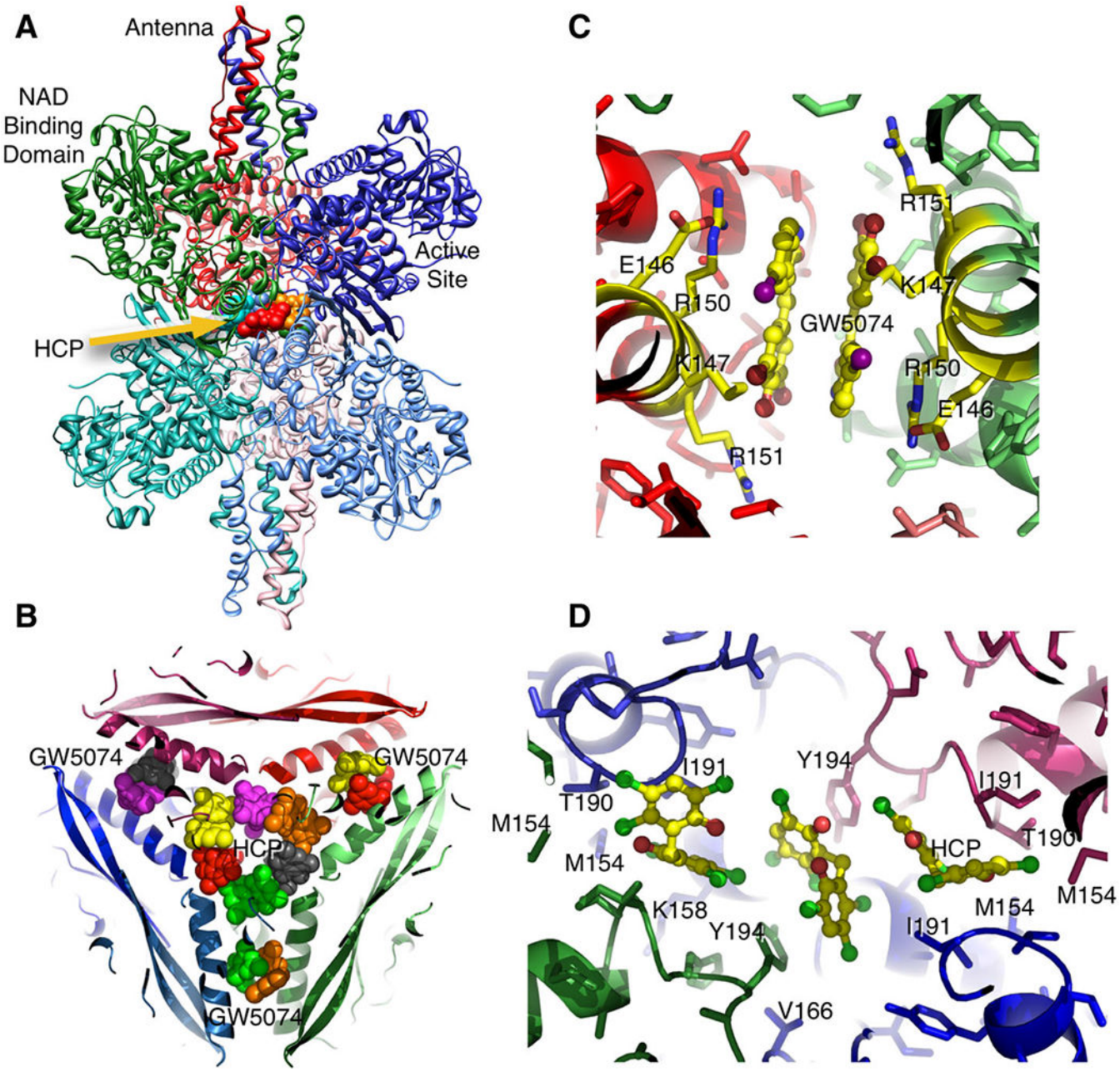

Fig. 5.

Binding sites for GW5074/bithionol and HCP [52]. **a** Ribbon diagram of GDH complexed with HCP. HCP binds as a ring inside the innermost core of the hexamer. **b**, **c** GW5074 and bithionol bind to the same location halfway between the core and the exterior of GDH as apposed to HCP (**b, d**) that binds to the innermost core

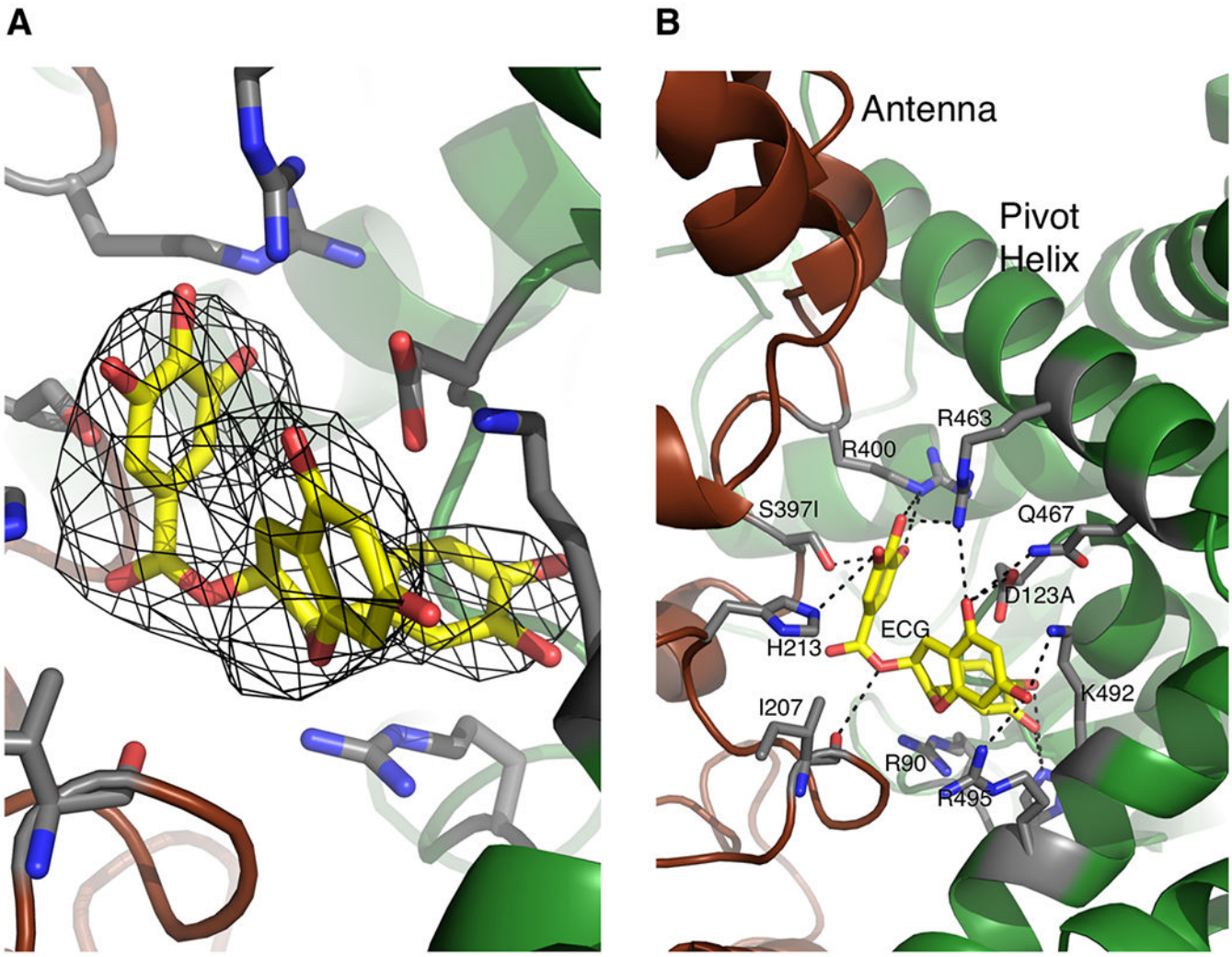

Fig. 6.

Location of the ECG/EGCG binding site [54]. **a** Sixfold averaged electron density of the bound ECG molecule. **b** Structure of ECG bound to GDH with the contact residues highlighted

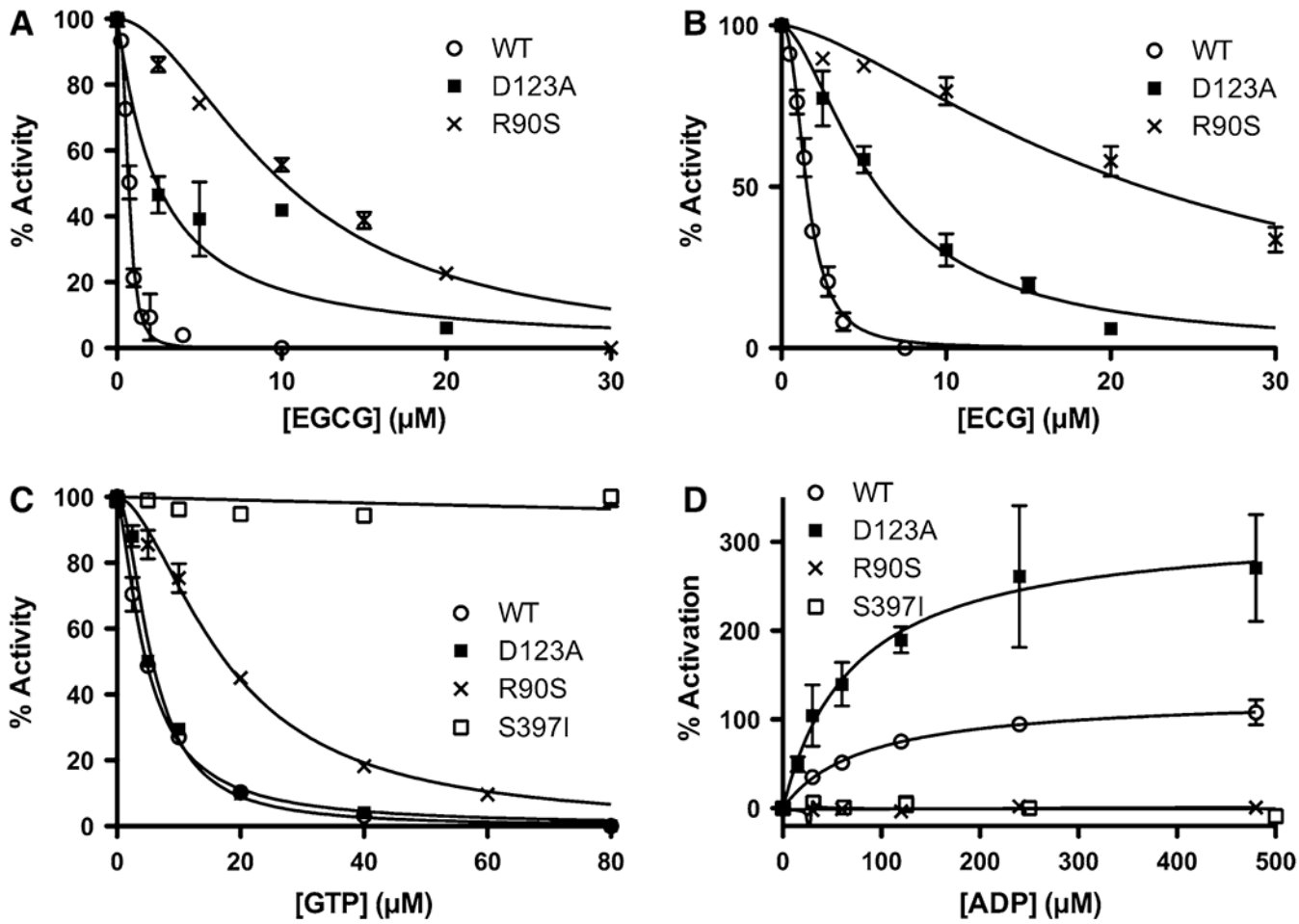

Fig. 7.

The effects of mutations in the ECG/EGCG binding site on EGCG inhibition (**a**), ECG inhibition (**b**), GTP inhibition (**c**), and ADP activation (**d**) [54]

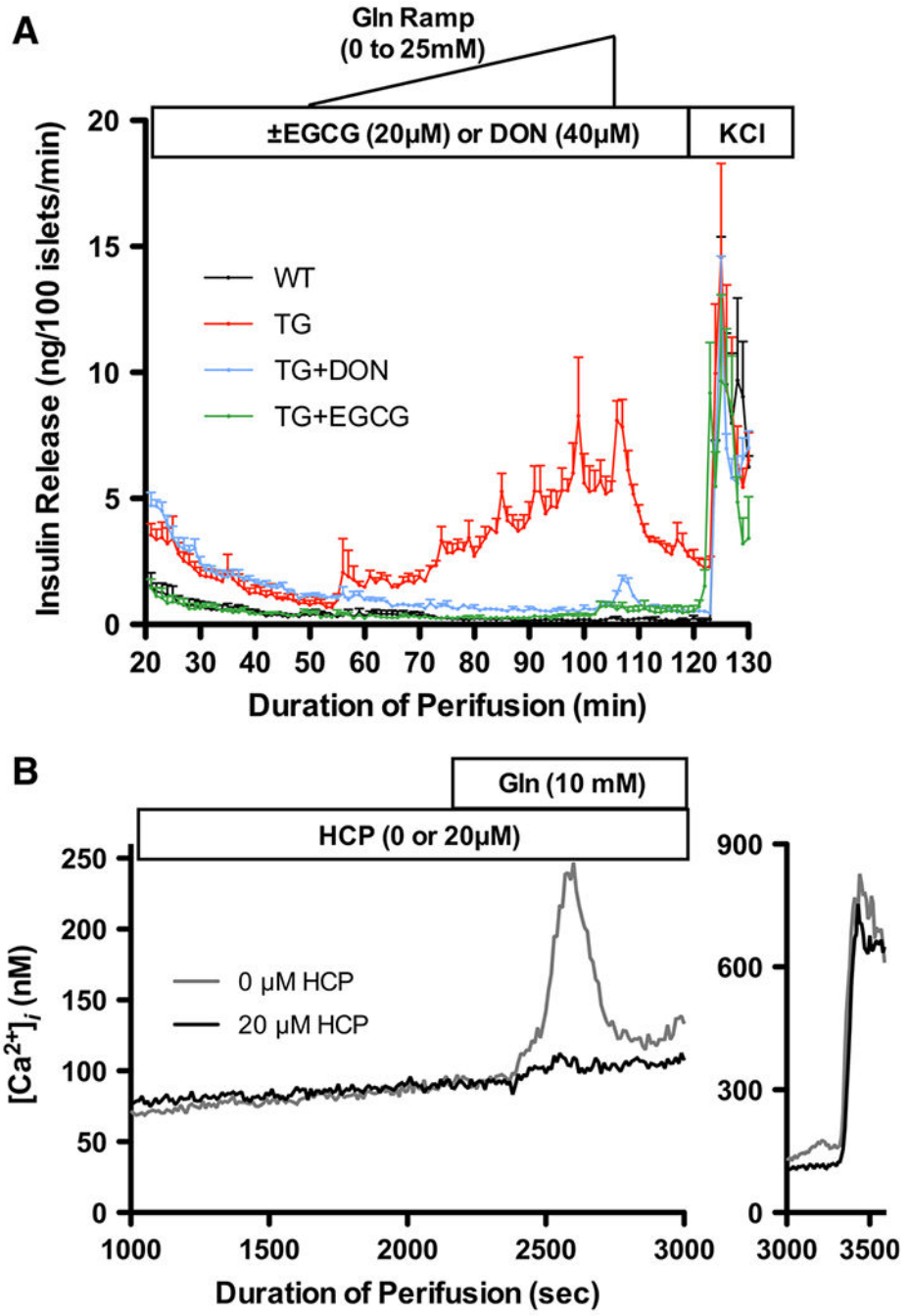

Fig. 8.

Effects of EGCG and HCP on pancreatic tissue from transgenic mice expressing the HHS form of human GDH [54]. **a** A glutamine ramp stimulates insulin secretion from transgenic mice (*red*) but not from wild type mice (*black*). Administration of EGCG (*green*) not only blocks this hyper-response, but also decreases the base level of insulin (levels at T = 0). In contrast, the glutaminase inhibitor DON (*blue*) blocks the hyper-response to glutamine, but does not ameliorate the higher basal serum levels of insulin. **b** HCP also blocks the hyper-

response to glutamine by the transgenic tissue. In this figure, the pulse of calcium signals the degranulation of the β-cells that is blocked by HCP

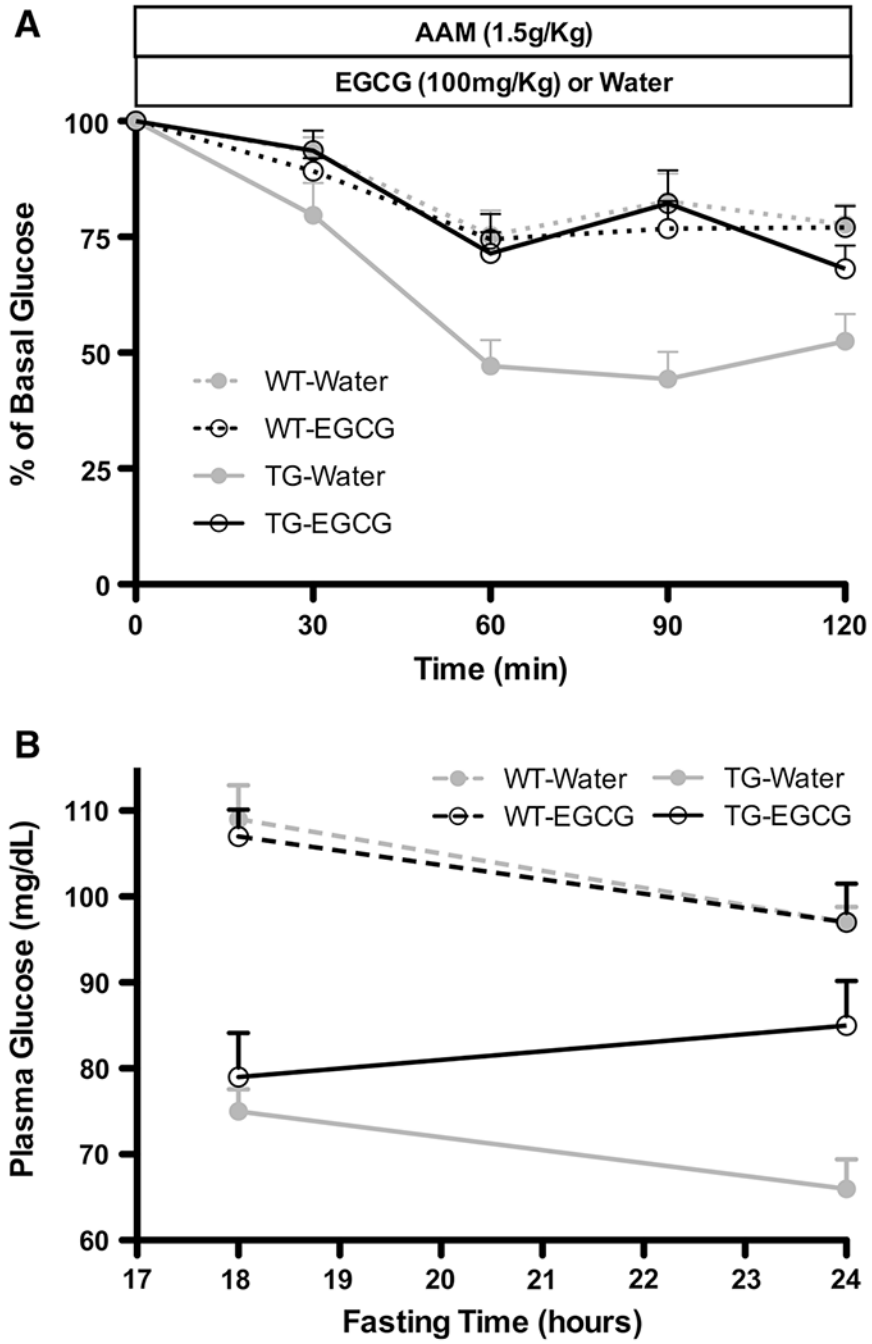

Fig. 9.

The effects of oral administration of EGCG on transgenic mice expressing the human HHS form of GDH. **a** When transgenic mice are administered an amino acid mixture, they hyper-secrete insulin and the serum glucose levels decrease. This is not observed in wild type mice and is blocked by administration of EGCG prior to amino acid challenge. Notably, EGCG has no effect on wild type mice, and is likely indicative of the normally tonic state of GDH.

b When EGCG is administered twice during fasting, an improvement in the basal level of glucose is observed