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Crystal structure of glutamate dehydrogenase 2, a positively selected novel human enzyme involved in brain biology and cancer pathophysiology

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; EDTA, ethylenediaminetetraacetic acid; hGDH, human glutamate dehydrogenase; MPD, 2-methyl-2,4-pentanediol; MWCO, molecular weight cut-off; NAD, nicotinamide adenine dinucleotide; NAD(P), nicotinamide adenine dinucleotide phosphate; PDB, protein data bank; PEG, polyethylene glycol; rmsd, root-mean-square deviation

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ABSTRACT

Introduction: Mammalian glutamate dehydrogenase (hGDH1 in human cells) interconverts glutamate to α -ketoglutarate and ammonia while reducing NAD(P) to NAD(P)H. During primate evolution, humans and great apes have acquired hGDH2, an isoenzyme that underwent rapid evolutionary adaptation concomitantly with brain expansion, thereby acquiring unique catalytic and regulatory properties that permitted its function under conditions inhibitory to its ancestor hGDH1. Although the 3D-structures of GDHs, including hGDH1, have been determined, attempts to determine the hGDH2 structure were until recently unsuccessful.

Hypothesis/aim: Comparison of the hGDH1/hGDH2 structures would enable a detailed understanding of their evolutionary differences. This work aimed at the determination of the hGDH2 crystal structure and the analysis of its functional implications.

Methods: Recombinant hGDH2 was produced in the *Spodoptera frugiperda* ovarian cell line Sf21, using the Baculovirus expression system. Purification was achieved *via* a two-step chromatography procedure. hGDH2 was crystallized, X-ray diffraction data were collected using synchrotron radiation and the structure was determined by molecular replacement.

Results: The hGDH2 structure is reported at a resolution of 2.9 Å. The enzyme adopts a novel semi-closed conformation, which is an intermediate between known open and closed GDH1 conformations, differing from both. The structure enabled us to dissect previously reported biochemical findings and to structurally interpret the effects of evolutionary amino acid substitutions, including Arg470His, on ADP affinity.

Conclusions: Our data provide insights into the structural basis of hGDH2 properties, the functional evolution of hGDH isoenzymes, and open new prospects for drug design, especially for cancer therapeutics.

Introduction

Glutamate dehydrogenase (GDH) reversibly converts glutamate to α -ketoglutarate and ammonia using NAD(P)⁺ as cofactors (Hudson & Daniel 1993). The enzyme is present in almost all living organisms, interconnecting carbon and nitrogen metabolism and contributing to energy homeostasis. Furthermore, it has a unique role in mammalian mitochondria, where it is highly abundant (Hudson & Daniel 1993; Cooper 2011). Inside the mitochondrial matrix, the ubiquitous mammalian GDH1 isoenzyme (hGDH1 designates the human version of the protein) produces α -ketoglutarate which subsequently feeds the Krebs cycle, serving anaplerotic functions and leading to the synthesis of ATP (McKenna *et al.* 1996). The metabolic role of mammalian GDH1 is tightly regulated, with its main allosteric activators (ADP, L-leucine) and inhibitors (GTP) reflecting the energy status of the cell (Plaitakis & Zaganas 2001).

Besides hGDH1 (encoded by the *GLUD1* gene), humans possess a second GDH isoenzyme, hGDH2 (encoded by the *GLUD2* gene), with markedly different functional properties (Shashidharan *et al.* 1994; Zaganas *et al.* 2012). hGDH2 emerged recently via retroposition during primate evolution, being only present in humans and some closely related great apes (Burki & Kaessmann 2004). The retroposition of the *GLUD1* gene that gave rise to the highly homologous *GLUD2* gene occurred less than 23 million years ago (Burki & Kaessmann 2004). After its emergence, *GLUD2* underwent rapid evolutionary adaptation, concurrently with primate brain development and perhaps, with evolving changes in other tissues where it is expressed (Vallender *et al.* 2008). This adaptation resulted in a significant divergence of its regulatory properties and its tissue and cell distribution patterns relative to the parental hGDH1 isoenzyme (Plaitakis *et al.* 2003).

Reflecting the very recent emergence of hGDH2 from hGDH1, the two human proteins show very high amino acid sequence homology (~97%), differing in only 15 out of 505 amino acids in their mature forms (Shashidharan & Plaitakis 2014). Despite this similarity, hGDH2 has unique enzymatic and regulatory properties. These include GTP resistance and low basal activity amenable to activation by ADP and/or L-leucine, lower optimal pH and relative sensitivity to thermal inactivation. These properties are to a large extent associated with only two of the 15 amino acid substitutions that occurred in the course of hGDH2 evolution (Zaganas *et al.* 2014). In particular, the Gly456 to Ala substitution confers GTP resistance, while the Arg443 to Ser change is associated with lower basal activity, though still permitting activation by ADP (Zaganas & Plaitakis 2002; Zaganas *et al.* 2002). In addition, the Arg443 change lowers the optimal pH and renders the enzyme heat-sensitive (Zaganas *et al.* 2014).

In addition, hGDH2 shows a unique tissue expression pattern compared to hGDH1. It is present in the human brain, kidney, testis and steroidogenic organs, but it shows little expression in the liver, where hGDH1 is pronouncedly expressed (Spanaki *et al.* 2010). Even in human tissues where the two isoenzymes coexist, their cellular distribution and inferred metabolic roles are often different (Zaganas *et al.* 2012). For example, in contrast to the mainly astrocytic localisation of hGDH1, hGDH2 is found in both human astrocytes and neurons, where it is thought to contribute to glutamate handling, both as a neurotransmitter and as a metabolic intermediate (Plaitakis *et al.* 2019). The importance of hGDH2 in human physiology and pathophysiology is further underscored by recent evidence concerning its putative role in early nervous system development (Li *et al.* 2016), neurodegenerative processes (Bao *et al.* 2009; Wang *et al.* 2014;

Plaitakis *et al.* 2010) and oncogenesis (Chen *et al.* 2014; Franceschi *et al.* 2018). Regarding its role in cancer pathophysiology, hGDH2 promotes tumor cell survival especially under deprived conditions, such as glucose or glutamine depletion (Takeuchi *et al.* 2018).

Each of the human GDH genes (*GLUD1* and *GLUD2*) encodes for a 558-residue protein. In both cases, the 53 N-terminal residues correspond to a leader peptide sequence, responsible for the transport of the proteins to the mitochondrial matrix where they become functional as hexamers (Kalef-Ezra *et al.* 2016; Kotzamani & Plaitakis 2012; Mastorodemos *et al.* 2009). Their mature forms result after removal of the leader peptide.

The 3D-structure of hGDH1 (Smith *et al.* 2002) and the structures of several other mammalian and non-mammalian GDHs (Smith *et al.* 2001) have been determined by X-ray crystallography. The hGDH1 structure is a symmetric homo-hexamer, with each subunit consisting of the N-terminal glutamate binding domain, the NAD⁺ binding domain, the antenna, the pivot and the C-terminal helices. On the other hand, despite several attempts, the crystallization of hGDH2 has remained elusive for several years. Here, we report the crystal structure of the hGDH2 protein determined at 2.9 Å resolution, after a new protein expression approach was introduced which allowed production of protein in mg quantities and growth of data-quality crystals. The functional implications of the structural findings are also reported.

Materials and Methods

Macromolecule production

For the construction of the hGDH1- Met415Leu/Arg443Ser/Gly456Ala/Arg470His quadruple mutant, a 351-base pair fragment encoding amino acids 379–495 of the mature hGDH2 peptide was cut from a *GLUD2* cDNA (subcloned in the pBluescript II SK(+) vector) using restriction endonucleases BsmI and BsrGI (New England Biolabs) and ligated into a similarly processed *GLUD1* cDNA, replacing the corresponding region. The hGDH1-Arg443Ser/Gly456Ala double mutant was made as previously described (Kanavouras *et al.* 2007). The resulting chimeric cDNAs (of the double and the quadruple mutant) were subcloned into a pVL1393 Baculovirus transfer vector and expressed using the BD BaculoGold™ system on Sf21 cells. Recombinant wild-type hGDH2 protein lacking the 53 amino acids of signal peptide was also produced in Sf21 cells using the Baculovirus expression system (Shashidharan *et al.* 1994) (**Table S1**). The human cDNA for hGDH2 was subcloned downstream of the p10 promoter of the plasmid vector pFastBacDual (RRID:Addgene_84869) (ATG-Biosynthetics, Germany). Recombinant pFastBacDual-*GLUD2* was used to transform flexiBacTurbo-containing *E. coli* (FBT) cells, containing a >135kb circular Baculovirus (bacmid). Positive colonies were selected for amplification and the bacmids were isolated and evaluated by PCR, using primers that bind at both sides of the insert. Finally, recombinant bacmids were used to transfect Sf21 cell cultures (ECACC Cat# 05030202, RRID:CVCL_JX37) (using the Cellfectin II reagent) in order to produce the P1 Baculovirus which was then used to upscale protein expression. This study was not pre-registered and no institutional ethical approval was required. Cell lines and plasmids will be shared upon reasonable request.

For the present study we used the commercially available Sf21 insect cell line, widely used in the expression of recombinant proteins. This cell line originally derives from the pupal ovarian tissue of *Spodoptera frugiperda*. To date, the latest version (version 10, March 25 2020) of ICLAC's Register of commonly misidentified cell lines, does not list Sf21 as such. Although we have not used STR-based authentication of our Sf21 cell line, there are strong indications that our cultures were pure and homogenous throughout the experiments: First, Sf21 is not a commonly misidentified cell line, according to ICLAC. Second, Sf21 cultures grow in very different conditions than the common mammalian cell lines, as they are kept in a 27°C incubator without CO₂ complementation, equipment dedicated only for this specific type of cells, and in the presence of non-mammalian culture medium (Grace's Insect medium). Thus, cross-contamination possibility with other cell lines was negligible. Nevertheless, as the Sf21 endogenous insect GDH enzyme is reactive with only NADH, whereas hGDH1, its mutants and hGDH2 are reactive with both NADH and NADPH, we have enzymatically tested purified fractions with both coenzymes. We have found that fractions reactive for both coenzymes, indicating hGDH activity, were separate from fractions reactive only to NADH, indicating insect GDH activity (Zaganas & Plaitakis 2002). Standard Sf21 cell line protocols, as provided by the manufacturer (ThermoFisher Scientific), were followed for the maintenance and growth of cells throughout the study. Cells were cultured initially as adherent and subsequently in suspension. Cell numbers were monitored at each passage, when they reached mid-log phase of growth, corresponding to 90% confluency for adherent, and 2×10^6 cells/ml cell density for suspension cultures. Generally, cells were passaged 2 to 3 times a week, up to a maximum number of 30 passages. Healthy culture samples were frozen at early passage (up to passage 10), and a fresh culture was started from frozen stocks approximately every 2 months.

The Sf21 cell line was grown in Grace's Insect medium supplemented with FBS and gentamicin. The cells were kept at 27°C. They were transfected with the viral stock when they were at the exponential phase of their growth (~75% confluency) and were incubated with this virus-containing medium for 4-5 days. The cells were then harvested, centrifuged and their pellets (containing the recombinantly expressed hGDH2 isoenzyme) were stored at -80°C for further use.

For cell lysis and homogenization, the cell pellets were re-suspended in lysis buffer containing Tris-HCl, NaCl, EDTA (pH 7.4), Triton 1% and protease inhibitors, and were processed with a tissue homogenizer. The obtained crude cell extracts were subsequently used for protein purification. Protein expression levels in the crude extracts were assessed by measurement of the enzyme-specific activity (measurement of GDH activity per mg protein of the extract) spectrophotometrically at 340 nm in Triethanolamine (TRA) buffer pH 8.0, using α -ketoglutarate, ammonia, NADPH and 1mM ADP or by Western Blot using the specific anti-hGDH2 antibody (Zaganas *et al.* 2012).

Following standard protocols, hGDH2, hGDH1-Arg443Ser/Gly456Ala double mutant and the hGDH1-Met415Leu/Arg443Ser/Gly456Ala/Met415His quadruple mutant were purified in a multi-step procedure which included two stages of ammonium sulfate precipitation, hydrophobic interaction chromatography and, hydroxyapatite chromatography (Zaganas & Plaitakis 2002): Initially, the supernatant of the cell lysate was subjected to a 30% ammonium sulfate precipitation, the resulting new supernatant was subjected to a 65% ammonium sulfate precipitation and the pellet was resuspended in a 15% ammonium sulfate solution (in Tris-HCl, pH 6.0).

For the first chromatography step, a hydrophobic interaction phenyl-sepharose column (GE Healthcare, UK) was used. The resin was equilibrated with a 15% ammonium sulfate solution before loading the cell extract sample. Elution was performed using a 15% ammonium sulfate solution-90% ethylene-glycol solution gradient. Verification of protein-containing fractions was performed by spectrophotometric measurement of the enzyme reaction.

The enzyme-containing fractions from hydrophobic interaction chromatography were pooled together and dialyzed against 200mM KCl solution, using a dialysis membrane with MWCO 7000 (Viscose, USA). The post-dialysis sample was then subjected to hydroxyapatite chromatography (Biorad, USA). The resin was equilibrated with 50mM sodium phosphate pH 6.8, before sample loading. The enzymes were finally eluted with a 50mM-400mM sodium phosphate pH 6.8 gradient. Fractions containing the purified protein were pooled and concentrated prior to subsequent crystallization and enzymatic studies.

Crystallization

Crystallization conditions for hGDH2 were screened using the hanging-drop vapour-diffusion method in 24-well Linbro cell-culture plates. The drops were made up of 2 μ l protein solution of 9.7 mg/ml mixed with an equal volume of reservoir solution and were equilibrated against 1000 μ l reservoir solution at 291 K. Initial crystallization screening was performed using commercially available kits including Structure Screen 1 and 2, Grid screen Ammonium Sulfate, Grid screen MPD, Grid screen PEG 6000, GBS screen 3, 6, 7, 8, 9, 10, Grid screen PEG ion, MEB FAK and Natrix screen, from Molecular Dimensions. Additional screening was performed using an OryXNano crystallization robot from Douglas Instruments, with the crystallization kits JBScreen JCSG++ HTS, Structure Screen 1, 2 MD1-30, 1-31, 1-32, 136 and MIDAS. Data quality crystals were obtained with 8% PEG 8000, 15% MPD, 0.4M NaCl and 0.1M phosphate buffer pH 7.0 (**Figure S4**). The concept of ionic strength reducers (Papanikolau & Kokkinidis 1997) was used for the refinement of the crystallization parameters. The crystallization information is summarized in **Table S2**.

Data collection and processing

X-ray diffraction data were collected from a single crystal using synchrotron radiation on the X06SA beamline at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland (**Figure S5**). Crystals were flash-cooled to 100 K in a nitrogen-gas stream using an Oxford Cryosystems device. Images corresponding to 360°, of 0.25° rotation each, were collected. X-ray diffraction data were recorded with an Eiger 16MX (133Hz) detector. The diffraction data were subsequently indexed, integrated and scaled with the XDS (Kabsch 2010) and CCP4 program suites (1994). The details of data collection and processing and the unit cell parameters are listed in **Table S3**. The crystals belong to space group C222₁. Matthews coefficient calculations suggest the presence of one hexamer in the asymmetric unit and a solvent content of approx. 59%.

Structural Determination and Refinement

The structure was determined by the molecular replacement method using Phaser (McCoy *et al.* 2007). The hexameric human apo-enzyme hGDH1 structure (PDB code 1L1F) was used as the search model. Solution was obtained only after

the removal of residues 396-446, from the antenna domain. After obtaining the initial density map the antenna helices were constructed by the PHENIX AutoBuild Wizard (Terwilliger *et al.* 2008). Subsequently, model building and refinement were carried out iteratively using the coot (Emsley *et al.* 2010) and PHENIX (Adams *et al.* 2010) programs, respectively. Maximum-entropy electron density maps were calculated by GraphEnt (Glykos & Kokkinidis 2000). The final refinement statistics are summarized in **Table S3**. The final model includes residues 8-501 of chain A, 11-501 of chains B and C, 8-503 of chains D and F, and 10-501 of chain E. The water structure modeled is reasonable for the 2.9 Å resolution, as 0.07 water molecules per protein atom were assigned, which is very close to the expected value (Carugo & Bordo 1999; Gnesi & Carugo 2017). The waters molecules included in the final model were stable in the crystallographic refinement. The positions of sodium and chloride ions are based on geometric/ chemical criteria and clear electron density peaks, however these ions are difficult to distinguish from waters.

The coordinates and structure factors have been deposited with PDB code 6G2U. Structural superpositions were performed by lsqkab (Kabsch 1976) from CCP4 suite of programs (Winn *et al.* 2011). Figures were prepared using PyMOL (DeLano 2010).

Enzymatic studies

Analysis of the enzymatic properties (ADP activation) of the quadruple hGDH1-Met415Leu/Arg443Ser/Gly456Ala/Arg470His mutant was performed alongside the wild-type hGDH2 and the hGDH1-Arg443Ser/Gly456Ala double mutant. Assays were performed, unless otherwise specified, by measuring enzyme activity spectrophotometrically at 340 nm in the direction of reductive amination in 50 mM Triethanolamine (TRA) buffer pH 8.0, 2.6 mM EDTA, using 8 mM α -ketoglutarate, 100 mM ammonia, and 150 mM NADPH as substrates and about 0.7-0.9 μ g of purified enzyme protein, as well as varying concentrations of ADP.

Results

Structure and assembly of hGDH2

hGDH2 was crystallized in the absence of allosteric regulators or active site ligands. The asymmetric unit comprises two trimers (chains A, B, D and C, E, F) of the enzyme (**Figure 1A**). Each monomer consists of the glutamate-binding domain (residues 1-210), the NAD⁺-binding domain (residues 211-399), the antenna (residues 400-448), the pivot helix (residues 449-478) and the C-terminal α -helix (residues 479-501), which are very similar to the structural features of hGDH1 (Smith *et al.* 2002) (**Figure 1B** and **Figure 2**). The antenna region is formed by the ascending and descending helix (**Figure 2C**). Local 3-fold axes, clearly recognizable in the self-rotation function calculated from the X-ray diffraction data (**Figure S1**), produce two crystallographically independent trimers (**Figure 1A**) which associate to form the biologically relevant hexameric assembly. Two nearly identical, crystallographically independent hexamers are formed, each as a homodimeric assembly of trimers (**Figure 1B**). These hexamers which are very similar to other known GDH1 structures, are assembled via crystallographic and local two-fold axes. The self-rotation function (**Figure S1**) reveals that

the two types of dyads are positioned in the ab-plane, being rotated relative to each other by 45°. The hexameric assembly is stabilized by an extensive network of interchain interactions among the glutamate-binding domains. A striking structural feature of the hexamer packing is a mixed, 10-stranded β -sheet formed by pairs of dyad-related monomers. In this sheet, interchain hydrogen bonds are formed between two antiparallel β 1-strands (**Figure 2B**) comprising residues 60-67. The hGDH2 trimers are stabilized through hydrophobic packing interactions in the core of an all-parallel 3- α -helical bundle formed by the ascending helices. Further interactions at the level of trimers include the interchain hydrogen bonds between the side chain of Ser455 (pivot helix) and the backbone nitrogen of Tyr405 (ascending helix) for the pairs of chains A/D, B/D, C/E and E/F; in addition, an interchain hydrogen bond is formed between the side chain of Glu439 (descending helix) and His412 (ascending helix) for chain pairs A/D, B/D and E/F.

There are extensive structural similarities between the six crystallographically independent hGDH2 monomers, both at the level of individual domains and the level of complete subunits. The least variable domain is the NAD⁺-binding domain with root mean square deviations (rmsd) of 0.26-0.41 Å for 188 C α atoms. The domain consists of a mixed, six-stranded β -sheet surrounded by α -helices (**Figure 2A**). The glutamate binding domain displays a higher degree of structural variability (rmsd 0.28-1.0 Å for 199 C α atoms). Except for monomer C, the domain is composed of a central highly twisted, five-stranded, mixed β -sheet which is surrounded by five α -helices (**Figure 2B**). Monomer C lacks strand β 5 and possesses a markedly shorter strand β 4. The highest variability among the six independent hGDH2 monomers is observed in the all- α -helical area of antenna and pivot helix (rmsd 0.34-1.8 Å for 78 C α atoms, **Figure 2C**). While the ascending and pivot helices have the same number of residues for all monomers, the descending helix is variable. The highest structural variability is observed in the tip (residues 425-431) of the ascending helix, which adopts three different conformations (**Figure 2C**).

Comparison at the level of complete monomers yields an rmsd of 0.28-1.0 Å for 490 C α -atoms, thus confirming the high degree of similarity of the six hGDH2 chains (**Figure 2D**).

From the analogy of hGDH2 to the structure of bovine GDH1 (boGDH1) in complex with glutamate (PDB code 6DHD), it can be inferred that the substrate binding pocket of hGDH2 is located in the interface between the glutamate- and NAD⁺-binding domains, comprising residues Lys94, Gly95, Gly96, Met115, Pro171, Asp172, Thr203, Asn353, and Val382. Interestingly, compared to hGDH1, the entrance of the substrate binding pocket is narrower in hGDH2 due to two amino acid substitutions, Ser174Asn and Ser331Thr. Thus, the decreased accessibility of this pocket could affect the observed lower basal activity of hGDH2.

Comparison with hGDH1

With 490 identical residues out of 505, hGDH1 and hGDH2 share a 97% identity in their amino acid sequences, and very similar 3D structures. As in the case of hGDH2, the six crystallographically independent monomers of the “open” conformation of apo-hGDH1 (PDB code 1L1F) have insignificant structural variations (Smith *et al.* 2002). Comparisons

(Figures S6 and S2) of the structures of the glutamate-binding, NAD⁺-binding and regulatory domains of hGDH1 with their counterparts in hGDH2 yields rmsds of 1.53-1.83, 1.42-1.48 and 1.3-1.95 Å, respectively.

The very similar NAD⁺-binding domains are characterized by the absence of strand β 3 in hGDH1, and by a higher helical content in hGDH1 compared to hGDH2 (Figure S6A, B). Strands β 3 and β 5 of the glutamate-binding differ slightly in their lengths between hGDH1 and hGDH2. Both strands are involved in the formation of the substrate binding pocket (Figure S6C, D). For the antenna and pivot helix area (Figure S6E, F), the ascending helix in hGDH1 is one residue shorter compared to hGDH2, while the descending and pivot helices are longer in hGDH1; the conformation of the hGDH1 antenna tip resembles more the antenna tip of the monomers A/E of the hGDH2 structure.

Structural alignment based on the glutamate domains of the hGDH1 monomers (Figure S2A) reveals that (i) the antenna and pivot helix area superimpose well, including the antenna tips which adopt the same conformation, (ii) on the other hand the orientation of the NAD⁺-binding domain varies among the six subunits (Smith *et al.* 2002).

Comparison with boGDH1

hGDH2 was also compared with boGDH1 (PDB code 6DHQ); this “closed” conformation has been associated with the presence of GTP (an allosteric inhibitor) and with abortive NAD(P)H-glutamate active site complexes (Smith *et al.* 2001). Structural comparisons between the domains of the closed boGDH1 conformation with their hGDH2 counterparts yielded rmsds in the range of 0.95-1.65, 0.57-0.64 and 1.9-2.61 Å, for the glutamate, NAD⁺-binding and antenna/pivot helix regions, respectively. The least variable NAD⁺-binding domain of boGDH1 has a slightly higher α -helical content and additional β -strand (β 7) in its central sheet. In the glutamate-binding domain length differences exist between the two structures in β -strands (β 3, β 5) which participate in the formation of the substrate binding pocket.

The antenna/pivot helix region of the two enzymes differ in the lengths of α -helices with the ascending and the pivot helices in boGDH1 being one residue longer than their counterparts in hGDH2, while the descending helix in boGDH1 is either equal or up to two residues shorter compared to its counterparts from the six hGDH2 monomers. The boGDH1 monomers have identical conformations in their antenna tips, resembling the antenna tip conformation of monomers B/C in hGDH2. Comparison of the hGDH2 trimers with those of the closed boGDH1 conformation, reveals that (i) the boGDH1 NAD⁺-binding domain is located closer to the glutamate domain, (ii) the pivot helix of boGDH1 rotates around its N-terminus moving its C-terminus away from the antenna and glutamate-binding domains, (iii) the antennas are differently orientated in the two structures.

hGDH2 adopts a semi-closed conformation

Earlier crystallographic studies of GDH1 have revealed a significant structural plasticity of the enzyme which adopts open and closed conformations (Smith *et al.* 2001; Smith *et al.* 2002). Structural comparisons at the level of complete GDH monomers (Figure 3) between hGDH2, and the open (apo-hGDH1) and the closed (ligated-boGDH1) states (PDB codes 1L1F and 6DHQ respectively) of GDH1 gave rmsds of 1.71-2.12 Å for the open conformation and 2.29-2.51 Å for the closed conformation (490 C α -atoms). Although several secondary structural elements of hGDH2 are well superimposable

with their counterparts from GDH1, other elements, e.g. the NAD⁺-binding domain adopt an intermediate conformation that lies between the open and the closed states (**Figure 3A**). The region comprising the antenna, pivot helix and the C-terminal helix (**Figure 3B**) has elements that superpose well in all three structures (C-terminal helix), while in the antenna helix hGDH2 has a higher degree of similarity to an open conformation. The pivot helix in hGDH2 adopts an intermediate orientation between open and closed conformations.

Overall, apo-hGDH2 adopts a semi-closed conformation, i.e. an intermediate state between open and closed GDH1 conformations. The transition from open to closed form via the semi-closed conformation, corresponds to a clockwise rotation of the NAD⁺-binding domain towards the glutamate-binding domain (Smith *et al.* 2001). This movement results in a narrowing of the active site pocket (**Figure 3A**), which possibly contributes to the lower basal activity of hGDH2 compared to hGDH1. On the other hand, beyond any effects from “open” and “closed” conformations, there is evidence that the activity of the enzyme is to a large extent determined by its structural flexibility, which allows it to undergo rapid turnover between the opening and closing of the catalytic cleft (Nassar *et al.* 2019).

Effects of the Gly456Ala substitution: Structural interpretation of the GDH2 resistance to GTP

Residue 456 from the pivot helix has a key role in the transition between closed and open conformations, a process which includes movements of the pivot helix and the NAD⁺-binding domain, leading to the opening of the active site cleft (**Figure 3**). Local flexibility is affected by an inter-subunit hydrophobic interaction at the base of antenna between residues Phe387 and Leu401 (**Figure 4F**). In GDH1, flexibility is also affected by the presence of the small and flexible Gly456 residue which packs against the Phe and Leu (**Figure 4F**). Replacement of Gly by the bulkier and less flexible Ala456 in hGDH2 is expected to reduce local flexibility, and thus to affect the opening and closing of the active site cleft.

Although GTP is an allosteric inhibitor for GDH1 the activity of GDH2, is not affected by its presence. Moreover, we have shown (Zaganas & Plaitakis 2002) that the Gly456Ala mutation is able to make hGDH1 resistant to GTP, and therefore the hGDH2 resistance to GTP can be attributed solely to Ala456. As it is shown in **Figure 4** (panels A, B and C), the GTP binding site is located in a pocket formed by the NAD⁺ binding domain and the N-terminus of the pivot helix. In addition, it is partly affected by a displacement of the C-terminus of the descending helix (**Figure 4**). Comparison of hGDH2 with the boGDH1 structure complexed with GTP, reveals that in hGDH2 the GTP binding site is occupied by Lys450 (**Figure 4C, E, F**). Furthermore, the site is surrounded by negatively charged residues making GTP binding electrostatically unfavourable. In particular, structural superposition based on the NAD⁺-binding domains reveals a movement of the pivot helix N-terminus towards the GTP binding site together with an approximately 20 degrees rotation (**Figure 4C**). As a consequence, the pair Lys446/Asp447, which is optimally located for GTP binding in boGDH1, is rotated 20 degrees anti-clockwise in hGDH2 and the equivalent Lys (450) overlaps with GTP (**Figure 4C, D, E**). During this movement, Asp451 (hGDH2) is found close to the GTP and together with Asp442 from the descending helix which is also moved towards the GTP site, establish an electrostatically unfavourable environment for GTP binding. As shown in **Figure 4F**, a reason behind these changes is the Gly456Ala substitution. The Ala456 methyl group acts as a wedge and

slightly pushes outwards the pivot helix resulting in the 20 degrees rotation of its N-terminus, which consequently moves Lys450 to the GTP binding site.

Effects of the Arg443Ser substitution on basal activity and heat sensitivity

Replacement of the GDH1 residue Arg443 by Ser in GDH2 was a key event early in the evolution of the *GDH2* gene in humans and great apes (Zaganas *et al.* 2014), which has been related with a lower basal activity and heat sensitivity (Zaganas *et al.* 2002). Arg443 is involved in the stabilization of open and closed conformations of GDH1. GDH1 structures reveal that the transition from open to closed conformations is associated with partial unfolding of the C-terminus of the descending helix (**Figure 3B**), immediately after residue Arg443, thereby reducing the length of the helix by almost a half turn. Arg443 forms conserved inter-subunit hydrogen bonds with backbone and/or side chains of Asp408 and Ser409 residues (**Figure 5A, B**) from the ascending helix of a neighbouring chain (Zaganas *et al.* 2002; Mastorodemos *et al.* 2015) and a conserved stacking interaction with Tyr405 (**Figure 5B, C**). Through these interactions, Arg443 establishes crucial inter-subunit interactions and connections that mutually stabilize the ascending and descending helices. In contrast, substitution of Arg443 by Ser in hGDH2 leads to a loss of the above interactions, and a looser packing in the antenna region (**Figure 5B, D**). This results in a higher flexibility in the area and in a lower stability of the enzyme (Mastorodemos *et al.* 2015), as we observed in earlier studies, which subsequently may contribute to a lower enzymatic basal activity and an increased heat sensitivity.

The ADP contribution to hGDH2 activity: The role of Arg470His change

ADP is an activator of hGDH1 and hGDH2 which binds only to the open state ((Plaitakis & Zaganas 2001). Comparison of hGDH1 (Smith *et al.* 2002) with the boGDH1 structures in the ADP-free (6DHQ.pdb) and the ADP-bound (6DHK.pdb) forms, reveals that all three structures are in the open conformation, with no significant structural changes induced by the binding of ADP. While several residues of the binding site interact with ADP in the open form, the closed conformation provides a less favorable environment for ADP binding. Inversely, ADP binding may alter system energetics and facilitate catalytic cleft opening as shown by dynamic simulation analyses (Nassar *et al.* 2019). In addition, removing the antenna lowers basal activity, but makes the hGDH1 enzyme extremely amenable to ADP activation, highlighting the role of the antenna on the catalytic turnover rate of the enzyme (Hoffpauir *et al.* 2019). The ADP binding site is located in a semi-exposed cleft in the interface between the NAD⁺-binding domain, the antenna/pivot helix region, the C-terminal helix and the glutamate-binding domain. This site is lined by secondary structure elements which undergo movements upon transition from the open to the closed conformation. In hGDH, ADP affinity is affected by the Arg443Ser change. As the hGDH1 Arg443Ser mutant has lower ADP sensitivity compared to hGDH2 (Zaganas *et al.* 2002; Plaitakis *et al.* 2003), we conclude that probably other evolutionary changes in hGDH2 partially compensate for the effects of the Arg443Ser substitution. In the semi-closed conformation of hGDH2, the ADP binding cleft comprises three residues from the pivot- and the C-terminal helices, with the following evolutionary substitutions relative to hGDH1: Gly456Ala, Arg470His and Asn498Ser. The location of Asn498Ser suggests that apart from marginal accessibility changes in the ADP binding cleft, only minor effects on ADP sensitivity should be expected. On the other hand, as already mentioned above, the Gly456Ala

substitution affects local flexibility and rigid-body movements of the pivot helix, a secondary structure element which is involved in conformational transitions of the enzyme. The reduction of local flexibility should affect the ADP binding affinity of the enzyme. Indeed, the double hGDH1 mutant Arg443Ser/Gly456Ala exhibits a reduced ADP sensitivity compared both to hGDH1 and to hGDH2 (Kanavouras *et al.* 2007). The decreased ADP affinity ($SC_{50} = 198.4 \pm 5.6 \mu M$, Figure 7) of the double mutant of hGDH1 (Arg443Ser/Gly456Ala) relative to hGDH2 ($SC_{50} = 98.9 \pm 4.2 \mu M$), suggests that in the hGDH2 molecule some of the evolutionary mutations counteract the effects of the Arg443Ser/Gly456Ala substitutions on ADP affinity. Subsequently, to determine the complete implications of evolutionary substitutions in the regulatory unit (antenna and pivot helix) on ADP affinity, we constructed and characterized the hGDH1 Arg443Ser/Gly456Ala/Met415Leu/Arg470His mutant (Figure 7). Relative to the double mutant Arg443Ser/Gly456Ala, the addition of the Met415Leu and Arg470His mutations results in an even more pronounced drop in ADP affinity ($SC_{50} = 453.1 \pm 35.1$). Met415Leu is unlikely to contribute significantly to this effect due to its remote position, as shown also by allosteric regulation analyses (Zaganas *et al.* 2002). Even though when introduced alone in the hGDH1 enzyme the Arg470His change does not significantly affect its behavior (Zaganas & Plaitakis 2002), it is mainly its structural and physicochemical properties of substitution that can adequately interpret the observed reduction of ADP affinity in the quadruple compared to the double mutant. The Arg470His substitution affects a cluster of positive charges (Arg/Lys) in the ADP binding cleft, located in the immediate proximity of ADP (Figure 6). Removal of Arg470 from this cluster is expected to weaken attractive electrostatic interactions with ADP and ultimately lead to a reduced ADP affinity. On the other hand, the His residue which replaces Arg470, is positioned significantly further from ADP and its charge strongly depends on conditions, e.g. pH.

Discussion

Despite the knowledge of the crystal structure of hGDH1 (Smith *et al.* 2002), the absence of an experimentally determined hGDH2 structure has considerably hindered efforts for an in-depth understanding of the evolutionary divergence between hGDH2 and its parental hGDH1 enzyme. Although hGDH2 shows a 97% amino acid sequence similarity to hGDH1, the importance of hGDH2 for human evolution is underscored by phylogenetic data showing that it evolved to the human lineage under positive Darwinian selection indicative of adaptive DNA sequence evolution. This adaptation occurred concomitantly with pre-frontal cortex expansion, equipping hGDH2 with novel abilities that permit amplification of the brain glutamatergic system (a crucial step in the development of the cognitive abilities). A most important novel property of hGDH2, conferred by the Gly456Ala evolutionary substitution (Zaganas & Plaitakis 2002), is the dissociation of its function from GTP control, a mechanism that largely determines glutamate flux through the ancestral hGDH1 pathway. Instead, hGDH2 activity is regulated by distinct molecular mechanisms with the Arg443Ser evolutionary substitution being the major player (Zaganas *et al.* 2002). The structural basis for these biochemical/functional properties was completely unclear in the absence of an experimental 3D-model of the protein. This gap has been now filled with the structure reported here. The use of the baculovirus system enabled hGDH2 overexpression in mg quantities led to the growth of data-quality hGDH2 crystals, while earlier attempts using other expression systems (*E. coli*) had failed to

produce satisfactory results. The obtained hGDH2 crystals were sufficient for structure determination of the enzyme at 2.9 Å resolution. It should be noted that, in contrast to hGDH1 which has a highly stable structure, hGDH2 is heat-labile and depends on protein concentration for its activity. Hence, inherent structural properties, e.g. structural lability, probably prevent diffraction of hGDH2 crystals to higher resolutions.

The hGDH2 structure adopts a semi-closed conformation that deviates both from the fully open conformation of apo-hGDH1 and the closed one of boGDH1. This structure explains several of function/ sequence relationships and the unique enzymatic properties of hGDH2 (Zaganas *et al.* 2009), including the effects of key mutations that occurred early in the evolution of the *GDH2* gene. It also helps explain the observation that in the absence of allosteric activators, an increase of phosphate buffer concentration results in an increased basal activity of hGDH2, while no such correlation exists for hGDH1 (Zaganas *et al.* 2009). The positions of twenty-three phosphate ions have been found in the hGDH2 crystal structure, fifteen of which occupy positions (**Figure S3**) which can be associated to earlier studies of GTP/GDH1 and ADP/GDH1 complexes: In all six monomers, one phosphate ion occupies the position of GTP γ -phosphate in GTP/GDH1 interactions, while in monomers A, B, E and F, a second phosphate occupies a position close to the position of GTP β -phosphate. Finally, except for E, all hGDH2 subunits bind a phosphate atom in the position where the ADP β -phosphate is observed in ADP/GDH1 complexes (**Table 1**). Comparison of hGDH2 with the hGDH1 structure in the presence of phosphates (PDB code 6DQG), reveals that in both cases the phosphate ions occupy nearly identical positions in the GTP and ADP binding pockets, being hydrogen-bonded with the pivot helix (residues Lys450 and Arg463) and a loop in the interface between the NAD⁺- and the glutamate-binding domains (residue His213). For these secondary structural elements, the open and semi-closed conformations are remarkably similar and deviate significantly from the closed conformation. Our hypothesis is that the phosphate ions bound at the GTP and ADP sites of hGDH1 and hGDH2 stabilize a conformation which is closer to an open one. An open structure of hGDH2 probably enhances the basal activity of the enzyme which is lower than that of hGDH1, possibly by increasing the accessibility of its glutamate binding site, which relative to hGDH1 is narrowed by residues Asn174 and Thr331 and the movement of the NAD⁺-binding domain. On the other hand, in agreement to experimental observations, the presence of phosphate ions, is not expected to significantly affect the basal activity of hGDH1 due to the higher accessibility of its substrate binding site.

The semi-closed conformation and the changes in the accessibility of the active site or the phosphate binding sites are important insights from the hGDH2 structure. Previous work by other authors has demonstrated that during each round of catalysis between an initial, unliganded open conformation to a final, ligand-bound closed one, GDH undergoes significant movements involving the NAD-binding domain (Nassar *et al.* 2019). Our results for hGDH2 suggest that a semi-closed initial conformation constrains the movement of the NAD-binding domain, making it thus "stiffer" compared to GDH1. The functionally important structural plasticity of GDH molecules is reflected in the new conformation adopted by hGDH2 which is undoubtedly unrelated to crystal packing effects, as the two crystallographically independent hGDH2 molecules are located in different crystalline environments, and yet exhibit very similar semi-closed conformations. Based on this, we propose that the low basal activity of hGDH2 is a combined effect of the semi-open conformation which limits the NAD-binding domain movements during the catalytic cycle and of glutamate binding site accessibility which is

affected by evolutionary amino acid substitutions in the hGDH2 molecule. It should be noted, that as we have already shown, the basal activity of hGDH2 is not negligible, but can reach up to 1/3 of that of hGDH1, depending on the enzyme concentration (Zaganas *et al.* 2009).

Conclusions

Our understanding of the emergence and evolution of the *hGDH2* gene from *hGDH1*, in response to selective pressures that relate to glutamatergic demands of the expanding ape brain (Zaganas *et al.* 2014; Plaitakis *et al.* 2017) has been until recently unsatisfactory, due to the absence of the 3D-structure of hGDH2. This was prohibitive for the development of structure-based interpretations of the evolutionary mutations affecting the properties of the new enzyme hGDH2. Our present work now provides the structural framework necessary for a detailed and comprehensive understanding of hGDH2 evolution.

The hGDH2 structure suggests that in the absence of allosteric or active site ligands, a semi-closed initial conformation can be established, which along with a reduced accessibility of the substrate binding pocket and a loosely packed antenna base contributes to the lower basal activity of hGDH2 relative to hGDH1. However, as a result of a considerable structural flexibility, this state is amenable to full activation as dictated by the metabolic needs of the cell. Our work provides also a rational structural interpretation of the observed properties of key evolutionary changes (e.g. Gly456Ala, Arg443Ser etc) that occurred in the transition from GDH1 to the GDH2 in modern apes (Burki & Kaessmann 2004), corroborating our earlier biochemical findings (Zaganas & Plaitakis 2002; Zaganas *et al.* 2002). Furthermore, the structure-based interpretation of the properties of a hGDH1 Met415Leu/Gly456Ala/Arg443Ser/Arg470His mutant, revealed the key role of the residue 470 for the ADP affinity of hGDH.

Interestingly, due to the extensive structural similarity of hGDH1 and hGDH2 which are often co-expressed in the same cell type (Plaitakis *et al.* 2019), the possibility of GDH hetero-hexamer formation from various combinations of the isoenzymes cannot be ruled out. Future investigations towards this direction will explore GDH oligomerization pathways.

In summary, the structure determination of hGDH2, is an important breakthrough in the efforts to understand GDH evolution, as well as the structure-function relationships governing GDH metabolic activity in humans. Given that hGDH1 has long been an attractive drug target, especially in relation to diabetes (Li *et al.* 2012; Li *et al.* 2009; Smith *et al.* 2019), the new structure can contribute the development of hGDH1- or hGDH2- specific molecules, that would be spared from off-target undesirable effects. In the last few years, hGDH2 has moved to centre stage concerning its role in cancer cell survival (Chen *et al.* 2014; Takeuchi *et al.* 2018; Spinelli *et al.* 2017; Franceschi *et al.* 2018; Shan & Lyssiotis 2017; Dang 2017), thus becoming an extremely attractive target for antineoplastic drugs. The structure reported here can help in advancing this goal through rational drug design.

--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

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Tables

Table 1. GDH binding sites for GTP, ADP and phosphates from crystallization buffer.

GTP binding site (boGDH1, 6DHM.pdb)	ADP binding site (boGDH1, 6NQT.pdb)	Phosphate binding sites (hGDH2, 6G2U.pdb)
<i>GTP γ-phosphate</i> Arg217 (221) Tyr 262 (266) Arg 265 (269) Arg 261 (265)		Arg 221 Tyr 266 Arg 269 Lys 450
<i>GTP β-phosphate</i> His 209 (213) Lys 446 (450) His 450 (454)		His 213 Lys 450
	<i>ADP β-phosphate</i> Ser 393 (397) Arg 396 (400)	Ser 397 Arg 400

Numbers in parentheses refer to structurally equivalent residues in human GDH (hGDH1).

FIGURE LEGENDS

Figure 1: Diagram of the hexameric hGDH2 molecule. (A) The six crystallographically independent subunits (monomers) of the protein are assembled in the asymmetric unit as a dimer of trimers, with a local two-fold axis (blue oval) being perpendicular to the plane of the page. Individual chains are represented by different colors. (B) The biologically relevant hexamer is formed as a dimer of two identical trimers related by a crystallographic (i.e. exact) dyad axis. The different colors designate the different structural and/or functional features of the protein i.e. the GLU binding domain (blue), the NAD binding domain (green), the antenna (orange), the pivot helix red and the C-terminal helix (yellow).

Figure 2: Structure of hGDH2 (6G2U.pdb) domains: (A) The NAD⁺-binding domain, with a central six-stranded β -sheet surrounded by α -helices. Small structural variations among the monomers are in the boxed areas. (B) The glutamate-binding domain with the long β -hairpin (β 1- β 2) involved in the formation of the hexamer core through hydrogen bonding

interactions with its counterpart from another monomer. Strand $\beta 5$ is missing in chain C. (C) Superposition of the six monomers of hGDH2 show the variability of the antenna tip region with three distinct alternative conformations. (D) Comparison of the hGDH2 after superposition of their glutamate domains (red). Conformational variability is insignificant for all domains.

Figure 3: Comparison of open, semi-closed and closed conformations from apo-hGDH1 (1L1F.pdb, chain A, yellow), hGDH2 (6G2U.pdb, chain A, pink) and substrate bound boGDH1 (6DHQ.pdb, chain A, blue) respectively. (A) Alignment of the three enzyme structures via superposition of the glutamate-binding domains; a clockwise movement of the NAD⁺ binding domain narrows the active site cleft. (B) Alignment of the structural features of antenna, pivot helix and C-terminal helix of the three conformations (open, semi-closed, closed).

Figure 4: Structural interpretation of the impact of the evolutionary mutation Gly456Ala on GTP resistance. (A) Superposition of hGDH2 (pink, PDB 6G2U) and boGDH1 (blue, PDB 6DHQ) complexed with GTP (space filling model) via their NAD⁺-binding domains. For clarity, only two monomers of each protein are shown. (B) The superposed hGDH2 and boGDH1 rotated relative to (A) by 90 degrees around the axis shown, to enable a clearer view of the GTP binding pocket. (C) Close-up of the GTP- and the potential GTP- binding sites of GDH1 and GDH2. The structural comparison reveals a displacement of the pivot helix N-terminus which results in a displacement of the GDH2 Lys450/Asp451 position compared to the equivalent pair of GDH1 Lys446/Asp447. (D) Although the GDH1 pair of Lys446/Asp447 is optimally located for GTP binding, the equivalent pair Lys450/Asp451 in GDH2 has moved in a way that Lys occupies the space where normally GTP is bound, and Asp has moved close to a position with an electrostatically unfavourable environment (E). Asp442 from the descending helix, which has moved towards the GTP binding site enhances the unfavourable electrostatic interactions. (F) The Gly456Ala substitution in GDH2 occurs in the core of these displacements with the methyl group of the Ala residue acting as a wedge in the hydrophobic core of Phe387/Leu401 pushing the N-terminus of the pivot helix outwards and the Lys residue towards the GTP binding site.

Figure 5: Structural interpretation of the impact of the Arg443Ser substitution. (A) Superposition of the hGDH1 (yellow, PDB 1L1F) and hGDH2 (pink, PDB 6G2U) trimers via their glutamate-binding domains. (B), (C) and (D) are close-ups of the environment of residue 443. The Arg443Ser substitution affects intersubunit hydrogen bonding (dashed lines in panel B) between residue 443 (monomer 1) and residues Tyr 405 and Ser 409 (monomer 2). Additionally, optimal Van der Waals interactions and a salt bridge between Arg443 and Asp408 are established in hGDH1 (panel C). Most of these interactions are lost in hGDH2 (panel D).

Figure 6: Changes induced by the Arg470His substitution in the ADP binding site of the Arg443Ser/Gly456Ala/Arg470His/Met415Leu mutant of hGDH1. (A) Comparison of hGDH1 (yellow) and hGDH2 (pink) dimers via superposition of their glutamate-binding domains. NAD is shown as a space filling model. (B) Close-up of the antenna and pivot helices. The four evolutionary mutations shown as sticks, ADP is depicted in form of a space filling model. (C) hGDH1 and hGDH2, each shown in two views related by a 90° rotation around the axis shown. (D)

Close-up of the pivot helix and the ADP binding site. (E) The ADP binding site. Lys and Arg residues are shown in green; if located on the pivot helix they are shown in more intense coloring.

Figure 7: ADP activation assays of purified wt-hGDH2 and mutant hGDH1 enzymes. Each point represents the mean of 3-5 determinations at a specific concentration, the error bars representing the standard deviation. The SC_{50} calculated was $98.9 \pm 4.2 \mu\text{M}$ for wt-hGDH2, $198.4 \pm 5.6 \mu\text{M}$ hGDH1-Arg443Ser/Gly456Ala double mutant and $453.1 \pm 35.1 \mu\text{M}$ for the hGDH1-Met415Leu/Arg443Ser/Gly456Ala/Arg470His quadruple mutant. Statistical significance of the difference in the SC_{50} using two-tailed t-test was at the level of $p < 0.001$ for the comparison of hGDH1-Met415Leu/Arg443Ser/Gly456Ala/Arg470His to both the hGDH1-Arg443Ser/Gly456Ala and the wt-hGDH2 enzymes, as well as for the comparison of hGDH1-Arg443Ser/Gly456Ala to wt-hGDH2.

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