Structural insights into the catalytic properties of GDH

Structural basis for the catalytic mechanism and α-ketoglutarate cooperativity of glutamate dehydrogenase

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ABSTRACT

Glutamate dehydrogenase (GDH) is a key enzyme connecting carbon and nitrogen metabolism in all living organisms. Despite extensive studies on GDHs from both prokaryotic and eukaryotic organisms in the last 40 years, the structural basis of the catalytic features of this enzyme remains incomplete. This study reports the structural basis of the GDH catalytic mechanism and allosteric behavior. We determined the first high-resolution crystal structures of glutamate dehydrogenase from the fungus Aspergillus niger (AnGDH), a unique NADP+-dependent allosteric enzyme that is forward inhibited by the formation of mixed disulfide. We determined the structures of the active enzyme in its apo form and in binary/ternary complexes with bound substrate (α-ketoglutarate), inhibitor (isophthalate), coenzyme (NADPH), or two reaction intermediates (α-iminoglutarate and 2-amino-2-hydroxyglutarate). The structure of the forward-inhibited enzyme (fiAnGDH) was also determined. The hexameric AnGDH had three open subunits at one side and three partially closed protomers at the other, a configuration not previously been reported. The AnGDH hexamers having subunits with different conformations indicated that its α-ketoglutarate–dependent homotropic cooperativity follows the Monod–Wyman–Changeux (MWC) model. Moreover, the position of the water attached to Asp154 and Gly153 defined the previously unresolved ammonium ion–binding pocket, and the binding site for the 2'-phosphate group of the coenzyme was also better defined by our structural data. Additional structural and mutagenesis experiments identified the residues essential for coenzyme recognition. This study reveals the structural features responsible for positioning α-ketoglutarate, NADPH, ammonium ion, and the reaction intermediates in the GDH active site.

Enzymes are important biological macromolecules and their catalytic functions govern a number of biological activities in all living organisms. Visualization of the active site of an enzyme-substrate complex or an enzyme bound to the catalytically competent reaction intermediate provides a direct proof of the reaction mechanism (1). Allosteric regulation of enzymes is one of the most fundamental processes that control several cellular activities. Obtaining quantitative molecular description of enzyme allostery has remained a central focus in biology (2). However, trapping the various structural intermediate states to gain detailed understanding about the kinetic properties of allosteric enzymes has remained very challenging (2,3). Glutamate dehydrogenase (GDH) is an oxidoreductase important for ammonia metabolism in archaebacteria, eubacteria, and eukaryotes (4,5). We have extensively studied this enzyme to discern the structural basis of unique properties related to its catalytic mechanism and allosteric behavior. GDH catalyzes the reversible oxidation of L-glutamate to α-ketoglutarate and serves as a coupler between carbon and nitrogen metabolism. Depending on
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the coenzyme specificity, GDHs can be classified as (a) NADP⁺-dependent, (b) NAD⁺-dependent, and (c) NAD⁺/NADP⁺-dependent (or dual specific) (6-8). Generally, the GDHs involved in ammonium assimilation are NADP⁺-specific, whereas the NAD⁺-dependent enzymes are involved in glutamate catabolism (9). The mammalian GDHs possessing dual-specificities can use either NAD⁺ or NADP⁺ with comparable efficiency and are allosterically regulated (10,11). NADP⁺ and NAD⁺ are identical except that NADP⁺ has an extra phosphate group attached to the 2'-hydroxyl of the adenosine. This specific difference is structurally remote to the reactive nicotinamide groups of these two coenzymes. Accordingly, the redox potentials of these two coenzymes are almost identical (8). Nature has developed multiple classes of GDHs which can efficiently discriminate between these two coenzymes; but how they accomplish this differentiation is unclear.

The NADP⁺-specific bacterial/fungal GDHs and the dual coenzyme specific mammalian GDHs are hexameric (12). The NAD⁺-dependent bacterial/fungal enzymes are either homo-hexamers (13) or homo-tetramers (14,15). Despite numerous studies in the last 40 years to identify the intermediates formed during the reaction catalyzed by GDHs (16-20), structural basis of the reaction mechanism of this enzyme remains unresolved. A number of medium/low resolution crystal structures of GDHs have been determined as complexes with substrates (α-ketoglutarate or glutamate) and coenzymes (NADP⁺ or NAD⁺) (7,12,13,16,21-23). However, these structures fail to explain the reaction mechanism and the role of different active site residues in catalysis, mainly due to their limited resolution and unavailability of any intermediate-bound, catalytically competent structures of this enzyme. The binding site of ammonia is yet to be identified in the GDH active site. Structural determinants for the recognition of NADP⁺ in NADP⁺-specific GDHs have remained ambiguous. Significantly, no representative structure of a fungal enzyme is available so far.

The filamentous fungi Aspergilli produce a NADP⁺-specific GDH involved in ammonium assimilation (24,25). Purified GDH from Aspergillus niger (AnGDH) (26) exhibits sigmoid saturation with α-ketoglutarate (Kₘ = 4.78 mM) and is competitively inhibited by isophthalate (Kᵢ = 6.9 μM). AnGDH shows a distinctive feature of unidirectional inhibition of forward activity by 2-hydroxyethyl disulfide (2-HED) modification without affecting its reverse activity (27). Surprisingly, the NADP⁺-dependent GDH from Aspergillus terreus (AtGDH) shows hyperbolic saturation with α-ketoglutarate (Kₘ = 6.0 mM) and its both forward and reverse activities remain unaltered in buffers containing 2-HED. Both AnGDH and AtGDH are expressed as polypeptide with 460 amino acids, share 88 % sequence identity (Fig. S1) and are active as hexamers (27). Despite their high sequence identity, AnGDH and AtGDH exhibit exceptional differences in kinetic properties; hence a structural justification is desired.

We report the first high-resolution crystal structures of fungal GDH. The structures of AnGDH were solved as apo-enzyme, catalytically competent ternary complexes, as well as complexes with reaction intermediates and an inhibitor. The structures of forward inhibited AnGDH (fiAnGDH) have also been determined as apo-enzyme and complexed with α-ketoglutarate (AKG). Analysis of structures complemented with functional characterization demonstrates the structural basis of the coenzyme specificity and kinetic cooperativity of this enzyme. Our data provide direct proof for some of the reaction intermediates of the catalytic mechanism. The results presented here are broadly applicable to all glutamate dehydrogenases (GDHs) studied so far, and some aspects extend to dehydrogenases in general.

RESULTS

Structural fold of AnGDH – We determined the first crystal structures of GDH from kingdom fungi (Fig. 1). The structures of AnGDH were solved as apo-enzyme as well as its complex with substrate (α-ketoglutarate)/coenzyme (NADPH), reaction intermediates (α-iminoglutarate (AIG)/2-amino-2-hydroxyglutarate (AHG)), fiAnGDH-AKG complex and inhibitor (isophthalate) at resolutions of 2.8, 1.8, 1.75, 2.25 and 1.9 Å, respectively (Table 1). The hexamer of fiAnGDH has three subunits complexed with α-ketoglutarate and the rest of the subunits in an unliganded form. All the structures are of high quality as reflected by their low R-factors and good stereochemical...
parameters (Table 1). Only the apo AnGDH structure has relatively high R-factors, mainly due to the lower resolution and poor redundancy of the diffraction data. All the bound ligands are unambiguously defined in the active site of AnGDH by clear electron densities. AnGDH has high sequence identity (Fig. S2) with other NADP+/NAD- specific GDHs and a few extra amino acid insertions. The overall structural fold of AnGDH is similar to the previously determined structures of E. coli GDH (EcGDH) and other GDHs (7,12,21-23). Each subunit of AnGDH consists of two domains separated by a deep cleft (Fig. 1a, b). Domain I is comprised of residues 1-190 and 437-460, and domain II consists of residues 191-436. These domains are mainly composed of α-helices and β-sheets which are numbered as H1-H16 and β1-β11, respectively. Domain I plays a significant role in subunit assembly and combining the protomers leading to formation of a hexamer with 32 symmetry (Fig. 1c). Domain I is mainly engaged in binding of α-ketoglutarate, whereas domain II facilitates binding of NADP(H). Domain II has seven α-helices and seven β-strands folded in a modified Rossmann fold (28,29) with β2-H8β3H9 and β11-H13β13H13 as first and second motifs, respectively. These two motifs are connected by H10-H10. Analysis of the domain II of AnGDH-AKG-NADPH complex shows that the residues present in the loops "β8-loop-β6" and "H5-loop-H10" are responsible for NADP(H) recognition (discussed in a later section). The interface between the two domains is formed by the interactions provided by long helices (H15 and H16). Like other GDHs, AnGDH forms a hexameric structure that may be considered to be composed of two trimers or three dimers. The dimeric interface is mainly formed by β1, β2, H1 and H16 of the substrate binding domain I, whereas the trimeric interface formation is mediated by the residues located at the N-terminal of H14 and the C-terminal of H15. Similar interfaces have also been reported for hexameric structures of EcGDH and P. falciparum GDH ( PfGDH) (9).

Conformational flexibility in AnGDH structure - Apo-AnGDH and fiAnGDH-AKG complex were crystallized with a hexamer in the asymmetric unit (Fig. 1c). Structural superpositions of both (Apo-AnGDH and fiAnGDH-AKG) show lower root mean square deviation (r.m.s.d.) values (0.6 Å) for equivalent monomers, indicating almost identical conformation of these two structures. AnGDH hexamer has a cylindrical shape with approximate height and diameter of 110 and 96 Å, respectively. Interestingly, each subunit of AnGDH hexamer has different conformation (Fig. 1d). Overall structural superpositions of A-subunit with B, C, D, E, and F subunits of AnGDH hexamer produced r.m.s.d. values of 0.8, 0.4, 2.2, 2.1 and 2.2 Å, respectively. Superposition of D-subunit on E and F produced r.m.s.d. values of 0.3 and 0.4 Å, respectively. Further, conformational differences among the subunits of the hexamer were also analyzed using the opening of the substrate/coenzyme binding cleft by measuring the distance (Table 2) (Fig. 1e) between the Cα atoms of Lys122 and Arg280. These results indicate that three protomers at one side of the hexamer are in an open conformation, and three subunits at the opposite side are in a closed one. Such symmetric opening and closing of trimers in a hexameric assembly of a GDH was observed for the first time. Interestingly, the closed subunits of apo AnGDH and only α-ketoglutarate bound form of fiAnGDH have identical cleft opening, indicating that the subunits in the AnGDH hexamer may remain in both open (three) and closed (three) states in the absence of any ligand. The opening of the binding cleft in AnGDH-AKG-NADPH complex is much smaller, with a distance of 6.0 Å (Table 2) (Fig. 1e, f) and similar values are also observed for other AnGDH ternary complexes reported in this study. Previously, the ternary complex structures of bovine GDH (21), B. thailandensis (BtGDH) and C. glutamicum (CgGDH) (30) have been reported with the opening cleft distances of 11.4, 9.9 and 9.4 Å, respectively. Hence, AnGDH ternary complex structure is the first representative with superclosed conformation (Table 2). The superposition of domain I (residues 2-190), domain II (residues 192-375) and hinge helix regions (residues 379-439) were performed separately and resulted in the r.m.s.d. values of 0.4, 0.3 and 1.0 Å, respectively. These values indicate very little structural modulation in the two domains; however, the hinge region undergoes a substantial conformational change. Although most of the residue positions remain unaltered, the side chains
of Gln12, Lys122, Arg193, Arg280, Arg407 and Arg418 adopt different conformations in the open and closed states of the AnGDH structures. The inter subunit salt bridge interactions (Arg407-Glu403, Lys171-Asp458) present between the open subunits within the hexamer are lost due to the domain closure. The domain movement in AnGDH analyzed by DynDom (31) web server indicates symmetric domain closure of 34˚ and rotation of domain II by 20˚ due to the conformational change in the hinge helices (H14 and H15) (Fig. 1f). The conformational flexibility of AnGDH structure plays an important role in the inter-subunit communication to control the substrate/coenzyme binding as well as the homotropic cooperative interactions with its substrate α-ketoglutarate (discussed in a later section).

Active site of AnGDH complexed with α-ketoglutarate and NADPH– The structure of ternary complex of AnGDH with α-ketoglutarate and NADPH has been determined at 1.8 Å; the electron density for the substrate and coenzyme in the active site was unambiguous (Fig. 2a). This complex represents the first non-mammalian GDH showing correct orientation of the adenosine 2'-phosphate group of NADPH in the catalytically competent enzyme active site. The domain closure facilitates appropriate positioning of NADPH and α-ketoglutarate as well as interactions of the ligands with active site residues. α-ketoglutarate is bound via several polar interactions involving residues Lys78, Gln99, Lys102, Lys114, Asp154, Arg193 and Asn346 (Fig. 2b and Fig. S3a). The distance between the reactive carbonyl carbon (C2) of α-ketoglutarate and hydride donating/accepting carbon (C4) of the nicotinamide group of the coenzyme in AnGDH is 2.8 Å, whereas it is 3.8, 4.11, and 4.14 Å, respectively, in the bovine GDH, BtGDH, and CgGDH. In AnGDH, a water molecule located close to the α-carbon atom of α-ketoglutarate forms a short hydrogen bond (1.9 Å) with the side chain of Lys114, implying its importance in catalysis. The coenzyme is held in the active site cleft via interactions with the residues primarily from domain II and a few others from domain I. The adenine ring is anchored inside a pocket formed by His84, Ile155, and Thr321 (Fig. 2c and Fig. S3b). The ribose of adenosine is placed in a groove formed by the side chains of Ser229, Asp252, and Ala320. The residues Gly228-Ala233 forming the GXGXXG/A motif (32) provide hydrogen bonding interactions to the coenzyme. Ser229, a part of this motif, forms a hydrogen bond with the 3'-hydroxyl group of adenosine. In order to position the 2'-phosphate group of NADPH, the Asp252 carboxylate group is pointing away from the ribose sugar and forms a salt bridge with the side chain of Lys277. In NAD'-dependent dehydrogenases, position of Asp252 is generally occupied by an Asp or Glu located at the C terminus of the second β-strand of the βαβ-fold; these residues form important hydrogen bonds with the 2'-hydroxyl group of adenosine (33,34). In AnGDH, the 2'-phosphate group is anchored primarily via direct hydrogen bonds with the side chains of Ser253, Gln282 of domain II, and Lys122 of domain I. Lys277 and His84 side chains also form water-mediated hydrogen bonding interactions with the 2'-phosphate group. Alanine mutants of these five residues were generated and the observed deviation of the measured kinetic parameters (Table 3, Fig. S4) of the mutants confirms involvement of these residues in NADPH binding. The alanine mutants of Ser253, Lys277 and Gln282, lose significant amount of the NADPH-dependent enzymatic activity as compared to the native enzyme. Notably, none of these mutants as well as the wild type enzyme show any measurable NADH-dependent activity. Because of the disruption of the polar interactions by alanine mutation of the polar residues, the apparent $K_m$ values for NADPH binding are increased to varying extents in the single mutants. The highest $K_m$ value was observed for Lys277Ala mutant which also showed weak positive cooperativity towards NADPH saturation. A significant decrease in $k_{cat}$ is observed for Ser253Ala and Gln282Ala mutants with latter having the lowest value. The catalytic efficiency ($k_{cat}/K_m$) of Ser253Ala, Lys277Ala and Gln282Ala has decreased drastically (220, 40 and 3300 times, respectively) as compared to the wild type enzyme. The catalytic efficiency lost in His84Ala and Lys122Ala mutants was 1.7 and 3.0 folds, respectively, and is not that significant. The measured kinetic parameters of the NADPH-dependent activities indicate primary involvement
of Ser253, Lys277 and Gln282 in binding the 2'-phosphate group of the cofactor.

The pyrophosphate group of NADPH is hydrogen bonded to the main chain of AnGDH GXGXXG/A motif (formed by residues Gly228-Ala233 of domain II) and is also hydrogen bonded with well-defined water molecules nearby. The 2'-hydroxyl group of the ribose sugar has hydrogen bonding interactions with the side chain of Arg82, Asp154, and Asn346 (Fig. 2c). A well-defined electron density (Fig. 2a-inset) indicates that the C4 atom of the nicotinamide ring has tetrahedral geometry, implying the presence of a reduced form of coenzyme in the enzyme active site. The nitrogen atom of the amide group is hydrogen bonded to one of the oxygen atom of the NADPH pyrophosphate moiety and the side chain of Asn231 (Fig. 2c). The distance between the α-carbonyl carbon (C2) of α-ketoglutarate and C4 atom of nicotinamide ring is 2.8 Å, indicating that reactive states of the substrate and coenzyme are trapped in the enzyme active site. Such a close interaction between the coenzyme and substrate has never been captured in crystal structures of GDHs reported previously.

Reaction intermediates in the AnGDH active site - The enzymatic reaction was carried out during the crystallization process following the scheme (Fig. 3a) in the presence of NADP+ so that α-iminoglutarate (AIG) formed in the enzyme active site does not get reduced to form L-glutamate. The high resolution (1.75 Å) electron density map (Fig. 3b-i, 3c) shows the presence of reaction intermediates and NADP+ in the active site. Initially NADP+ and α-iminoglutarate were refined (Fig. 3b-ii) in the active site. However, a positive residual $F_o-F_c$ electron density (Fig. 3b-ii) remained connected with α-iminoglutarate. Refinement of 2-amino-2-hydroxyglutarate with partial occupancy could satisfy the remaining positive electron density (Fig. 3b-iii).

This complex presents the first structural proof of formation of the α-iminoglutarate and 2-amino-2-hydroxyglutarate as intermediates during the reaction catalyzed by a GDH. Notably, the binding mode of α-iminoglutarate and α-ketoglutarate is identical. The α-imino group has polar interactions with the carboxylate group of Asp154 and the main chain carbonyl group of Gly153 (Fig. 3d and Fig. S5b). The α-hydroxyl group of 2-amino-2-hydroxyglutarate is hydrogen bonded to the Lys114 side chain (Fig. 3d and Fig. S5a). The carbonyl group of Gly153 adopts two alternate conformations in this structure. The amino group of Lys114 side chain acquires different conformations in AnGDH-AKG-NADPH and AnGDH-AIG-NADP+. The distance between the α-carbon atom of α-iminoglutarate and C4 atom of the nicotinamide moiety of NADP+ in AnGDH-AIG-NADP+ structure is 3.0 Å. The binding mode of NADP+ in AnGDH-AIG-NADP+ complex is almost identical as observed for NADPH in AnGDH-AKG-NADPH complex. The only difference is that in the α-iminoglutarate bound structure the 2'-phosphate group has moved closer to Lys277 and the side chain of this residue directly interacts with the phosphate oxygen atom (Fig. S5c). These structural data support the accommodation of the tetrahedral intermediate for the first time; an entity postulated before but with no such direct structural evidence. Our data directly implicate the formation of an α-iminoglutarate and 2-amino-2-hydroxyglutarate as the reaction intermediates in the catalytic mechanism of glutamate dehydrogenase, as invoked previously using spectroscopic analysis (13,17,19,20).

Binding mode of an inhibitor isophthalate in the AnGDH active site – This is the first structure of an isophthalate (IPT)-bound GDH (Fig. 4a). In fact, there is no isophthalate complexed protein structure available in the PDB. Isophthalate occupies the same binding pocket where α-ketoglutarate binds in the AnGDH active site (Fig. 4b and Fig. S6). All eight carbons of isophthalate are present in the plane of the benzene ring, but the oxygen atoms of the carboxylate groups are out of this plane. In contrast, the crystalline form of free isophthalate is reported to form a planar structure with all the atoms residing in one plane (35). The two carboxylates of isophthalate make identical interactions as those observed for α-ketoglutarate. The active site bound NADPH has an identical conformation in both AnGDH-AKG-NADPH and AnGDH-IPT-NADPH complexes. While the two carboxylate groups of the substrate and the inhibitor occupy conformationally similar positions, their other carbon atoms do not.
Interestingly, due to the presence of a hydrophobic inhibitor, the water molecule observed close to Lys114 in the AnGDH-AKG-NADPH is displaced in the AnGDH-IPT-NADPH complex. Notably, isophthalate has caused approximately 58.9° rotation of the peptide bond between Gly153 and Asp154 as compared to the α-ketoglutarate bound structure (Fig. 4b). The conformational change of Gly153 main chain carbonyl group is enough to position the aromatic ring of isophthalate in the binding pocket, suggesting the plasticity of the AnGDH active site. This plasticity may be essential for binding the reaction intermediates during catalysis.

**Hexameric structure of fiAnGDH with open and partially closed subunits**—In the hexameric fiAnGDH structure, three monomers (of one trimer) are in partially closed conformation and complexed with α-ketoglutarate. The remaining unliganded three subunits (of other trimer) have open conformations (Table 2). The covalent modification of Cys141 in all six subunits is clearly visible in the electron density map (Fig. S7). The α-ketoglutarate molecules bound in the active site of partially closed fiAnGDH subunits have different conformations when compared to those observed in AnGDH-AKG-NADPH complex (Fig. 5a, b). The striking conformational difference is seen for α-carbonyl group which mainly forms hydrogen bonds with the side chain of Lys114 and main chain -NH group of Gly80 in fiAnGDH-AKGP complex, whereas in AnGDH-AKG-NADPH complex this group is primarily interacting with the side chain of Asp154 as well as the main chain carbonyl group of Gly153. The coenzyme group of Asp154 interacts with the side chains of Arg82. The spatial arrangement of the AnGDH active site suggests that Arg82 might play a crucial role in maintaining the ionization state of Asp154. Arg82 is highly conserved among NAD⁺- and NADP⁺-dependent GDHs (Fig. S2). The $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values for NH₄⁺ binding to Arg282Gln mutant are 22.3 ± 1.1 mM, 10.3 ± 0.5 s⁻¹ and 4.6×10⁻¹ ± 0.02, respectively (Fig. S8). For the native enzyme, the $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values for NH₄⁺ are 1.4 ± 0.2 mM, 106.5 ± 0.9 s⁻¹ and 76.1 ± 9.3, respectively. Almost 16 folds increase in $K_m$ value and 165 times decrease in catalytic efficiency of Arg82Gln mutant is consistent with the role of Arg82 in AnGDH catalysis as proposed above.

In the AKG bound fiAnGDH monomers, an active site water molecule is visible and it is hydrogen bonded to the carboxylate group of Asp154 and main chain carbonyl group of Gly153 (Fig. 5a). This water molecule has not been observed before in any of the available GDH structures. Position of this water defines the space needed for ammonia binding during the catalytic conversion of α-ketoglutarate to L-glutamate (see Discussion).

**Intersubunit interactions in AnGDH structures**—Kinetic measurements of AnGDH showed sigmoidal saturation with α-ketoglutarate whereas hyperbolic saturation with NADPH (Fig. 6a, b). We performed careful analysis of the hexameric structures of this enzyme to decipher the structural basis of this allosteric feature. It is evident (Table 2) that individual subunits undergo conformational changes in the presence and absence of substrate, coenzyme, and ligand. During the catalytic cycle, domain I of each subunit of the hexameric assembly remains almost unchanged. However, domain II moves closer to domain I and it undergoes clockwise rotation (around 20˚) with respect to the three-fold axis of the hexamer. The structural arrangement suggests that only domain II facilitates interactions with the neighboring hexamers. Because of closure and rotation of domain II, the inter-subunit interactions are different in closed subunits as compared to the open ones.

Each subunit of the central hexamer has three-fold symmetry related lateral interactions with two neighboring subunits from different hexamers present in the same horizontal plane (Fig. 6c-e). On one side (with A, B, and C subunits) of the hexamer, the lateral interactions are mediated by the side chains of Asn335 and the main chain carbonyl group of Thr362. On the other side of the hexamer (with D, E, and F subunits), the H13 helix (residues 351-363) of one monomer is packed in the groove formed by the H10 helix (residues 283-287) and β10 (residues 298-302) from a subunit of the neighboring hexamer through hydrophobic and van der Waals interactions. The methyl groups of Thr356 and Thr362 side chains from one subunit have hydrophobic contacts with the side chains of...
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Ala302 and Cβ carbon atom of Ser285, respectively of the other subunit. Therefore the lateral interactions are mediated by Ser285, Ala302, Asn335, Thr356 and Thr362.

The inter-subunit interactions between the hexamers in the vertical directions are asymmetric (Fig. 6c, f, g). Most of the contacts are mediated by the residues from domain II of A subunit from one hexamer as well as D and E subunits of two other hexamers. The side chains of Glu217 of A subunit of central (first) hexamer forms a salt bridge interaction with the side chain of Lys299 of E subunit of the second hexamer. The side chains of Lys339 of A subunit also form hydrogen bonding interaction with side chain of Asn296 of E subunits of the second hexamer. Gln216 of A subunit makes hydrogen bonding interactions with the side chain of Lys299 and main chain of Ile259 and Val310 from E subunit. The loop region containing residues Ile259-Gly263 of E subunit of the second hexamer is also in close proximity of Thr213 from H7 of subunit A of central hexamer. On the other side of domain II of A subunit, Lys297 forms salt bridge interactions with Glu262 from the D subunit of the third hexamer. Ser246 side chain of A subunit also forms hydrogen bond with the side chain of Lys312 from D subunit of the third hexamer. Domain II of the B subunit of the central hexamer has only two polar interactions mediated by the side chains of Gln216 and Ser218 with the side chains of Asn214 and Gln216, respectively, from the F subunit of fourth hexamer. Domain II of the C subunit of the central hexamer does not have any interaction in the vertical direction with other hexamers. The difference in the extent of interactions with monomers in the vertical direction is mainly due to the closure and rotation of domain II. Thus, the residues primarily involved in vertical contacts are Gln216, Glu217, Ser246, Glu262, Asn296, Lys299 and Lys312.

Analysis of the crystal packing of the AnGDH ternary complex (AnGDH-AKG-NADPH) revealed that the crystallographic hexamers are composed of symmetric identical monomers arranged in the crystal with a large (104 Å diameter) solvent cavity (Fig. S9). The ternary complexes in the crystallographic hexamer, are in the super-closed conformations (Table 2) and have only diagonal inter-subunit interactions. Each monomer of a hexamer is interacting with a monomer from other hexamer by salt bridge interactions mediated by the side chain of Lys299 and Glu262. However, two inter-subunit salt bridge interactions (Arg407-Glu403, Lys171-Asp458) between the open subunits within a hexamer are lost because of the domain closure. Formation and disruption of several interactions among the subunits might play a crucial role towards the allosteric property of AnGDH.

DISCUSSION

GDH is an essential enzyme in all living organisms. Despite extensive studies (17,30) in the last 40 years on prokaryotic as well as eukaryotic GDHs, the structural basis of the coenzyme specificity and the mechanistic features of this enzyme remained incomplete. Also no structural information was available for a fungal enzyme. This prompted us to perform structural studies on A. niger GDH (AnGDH). Another reason for this study was to understand the structural basis of cooperative nature of this fungal enzyme.

This study presents the high resolution crystal structure of substrate and cofactor bound GDH Michaelis-Menten complex. We report the first fungal GDH structure. The hexameric AnGDH is unique with three open subunits at one side and three partially closed protomers at the other one. Such hexameric GDH structure has not been reported before. Partially closed subunits bind the substrate α-ketoglutarate. The position of the water attached to Asp154 and Gly153 defines ammonium ion binding pocket, which had remained unresolved. The binding pocket for the 2'-phosphate group of the coenzyme is better defined by our structural data. The structure of AnGDH-AKG-NADPH complex provides a glimpse of the super-closed catalytically competent enzyme with substrate and coenzyme at a favorable distance for hydride transfer. The structure of AnGDH-AIG-NADP+ complex captures the formation of α-iminoglutarate and 2-amino-2-hydroxyglutarate during the reaction. AnGDH-IPT-NADPH complex is the first structure of a protein-isophthalate complex and reveals the plasticity of the enzyme active site. Implications of the elucidated structures to the GDH reaction mechanism, coenzyme recognition and substrate cooperativity are discussed below.
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**Structural basis of α-ketoglutarate cooperativity in AnGDH**—Our structural data provide the possible explanation of the α-ketoglutarate-dependent cooperativity in AnGDH. It is evident that due to the inter-subunit interactions, the protomers of a catalytically incompetent hexamer are locked either in an open or partially closed conformation. In the super-closed conformation, the enzyme gains a catalytically competent form. These different conformational states can be directly correlated to the cooperative behavior of AnGDH. The Monod-Wyman-Changeux (MWC) model (36) and the Koshland-Nemethy-Filmer (KNF) (37) model are the two generally accepted models used to explain the kinetic cooperativity in a multimeric allosteric enzyme. According to the MWC model, the subunits of the enzyme are present in a reversible equilibrium between a low-affinity tensed (T) and a high-affinity relaxed (R) state in the absence of the ligand. When added, the ligand would bind to the R-state and the equilibrium will adjust by converting more of the T-state subunits to the R-state, therefore leading to positive cooperativity. On the other hand, the KNF model indicates that ligand binding to one subunit can induce conformational changes in the other subunits resulting in an increase in ligand affinity (and hence positive cooperativity).

Analysis of the structural data collated so far suggests that the unliganded structure of apo-AnGDH represents the low affinity state (resting-state), in which half of the hexameric enzyme (one trimer) remains as open/partially closed conformation (Fig. 7). Another low affinity form (resting-state) of the enzyme with all its six subunits in the open conformation may well exist; but we have not encountered such a structure so far. The hexameric unit with a trimer of three α-ketoglutarate-bound, partially closed subunits at one side and the trimer with three open subunits on the other - represents the T-state and is also not catalytically competent. Closing and opening of one trimer subunits at a time also correlates well with the measured Hill coefficient of 2.5 for α-ketoglutarate saturation (26). Binding of α-ketoglutarate and NADPH to all six subunits would generate a fully active enzyme species (R-state); this is consistent with the biphasic kinetic response to incubation with the product NADP⁺ and higher initial velocities with prior incubation of AnGDH with α-ketoglutarate and NADPH (26). Therefore, the homotropic α-ketoglutarate interactions of AnGDH may possibly be described by the mechanism (Fig. 7) following the MWC model.

Our data present the first structural evidence for the allosteric regulation in smaller GDHs. Based on the crystal structures of bovine GDH, the allosteric regulation in additional antenna domain containing mammalian GDH has been previously reported to follow the KNF model (38). The MWC allosteric model observed in AnGDH may be applicable to smaller GDHs without antenna domain, as this model nicely correlates with the measured Hill coefficient of 5.9 (39) of CsGDH. This implies that collective conformational transition of all six subunits in the hexamer contributes towards CsGDH positive cooperativity. The amino acid residues engaged in forming inter-subunit contacts in the AnGDH hexamer are replaced with other residues in AtGDH (sequence alignment; Fig. S1). Such interactions may account for the absence of α-ketoglutarate cooperativity in AtGDH. A systematic mutation analysis of these residues is expected to convert an allosteric AnGDH into a Michaelian enzyme and allow further probing into the mechanistic details of the allosteric regulation in this enzyme as well as other smaller GDHs.

**Structural basis of NADP(H) recognition by AnGDH**—The precisely defined position of the coenzyme in the high resolution electron density map of our AnGDH structures rationalizes the structural basis of coenzyme recognition in this enzyme as well as in other NADP⁺-dependent GDHs. Structural and mutagenesis data indicate that Lys122, Ser253, Lys277 and Gln282 are the primary determinants for coenzyme binding in AnGDH. In the AnGDH complexed structures, Lys277 side chain makes a direct (Fig. 8a) or water mediated (Fig. 2c) contact with the 2'-phosphate group of coenzyme. This observation suggests that the ionization state of the 2'-phosphate group of NADP(H) might dictate its interactions with these residues (Fig. 8a, b).

In the NADP⁺ bound AnGDH-AIG-NADP⁺ complex structure, the 2'-phosphate group might be dibasic (Fig. 8b); conversely, in other coenzyme bound structures this group might be monobasic. Lys277 is conserved in all the
NADP⁺-dependent GDHs (Fig. 8c). Interestingly, only Lys277Ala AnGDH mutant shows mild positive cooperativity towards NADPH, whereas the other mutants and wild type enzyme show no such kinetic behavior. This result makes Lys277 of AnGDH a unique residue governing the binding of 2'-phosphate of NADP(H).

One of the oxygen atoms (O2) of the 2'-phosphate group might remain neutral in both forms due to its close proximity to the Asp252 side chain (Fig. 8a, b) which is possibly negatively charged as it interacts with the side chains of Gln282 and Lys277. Although in AnGDH the Asp252 carboxylylate group does not interact directly with the phosphate group, it might still play a crucial role in positioning Gln282 and Lys277 side chains (Fig. 8a). In AnGDH, Ser253 and Gln282 side chains form hydrogen bonds with O2 oxygen atom due to its close proximity. Similar interactions of NADP⁺ 2'-phosphate group oxygen atom with threonine and arginine side chains are also observed in L. lactis 6-phosphogluconate dehydrogenase (LIPGDH) (40).

Both Asp252 and Ser253 of AnGDH are strictly conserved in the NADP⁺-dependent GDHs (41). Asp252 is the signature C-terminal end residue of the second β-strand of the βαβ-fold (Fig. 8d); and its negatively charged side chain has been so far believed to destabilize the binding of NADP⁺ 2'-phosphate (42, 43). Our results depict the essentiality of Asp252 for optimal orientation of Lys277 and Gln282 which facilitate NADP⁺ binding. Presence of Lys122, Lys277 and Gln282 creates a positively charged surface (Fig. S10) for binding the 2'-phosphate of NADP(H) in AnGDH. In other NADP⁺-dependent GDHs, this Gln282 is replaced by an amino acid residue with a proton donor -NH group (Fig. S2). The lowest catalytic efficiency of AnGDH Gln282Ala mutant points to the importance of this residue in coenzyme recognition. In EcGDH (12), CbGDH (30), the side chain of an arginine residue might serve the same purpose.

The other oxygen atom (O4) of the 2'-phosphate group should remain negatively charged and form an ionic interaction with a suitable residue on the protein. The Lys122 of AnGDH satisfying this ionic interaction is highly conserved in NADP⁺-dependent GDHs. The similar role for an equivalent lysine residue in CgGDH has also been proposed (30,44). The O3 oxygen atom of the 2'-phosphate may remain neutral or be negatively charged. Existence of two different ionization states of the 2'-phosphate group of NADP(H) was proposed before (32) but without any relevant structural data. The interactions present in the NADP⁺ and NADPH bound AnGDH structures provide the first direct evidence justifying the proposed ionization states of the 2'-phosphate group. However, further high-resolution structures, systematic mutagenesis studies, kinetic parameters measurements and quantum mechanics/molecular mechanics (QM/MM) calculations would prove valuable.

**GDH reaction mechanism: Conversion of α-ketoglutarate to L-glutamate** – The conversion of α-ketoglutarate to L-glutamate by glutamate dehydrogenase (GDH) takes place in sequential steps as suggested through the extensive biochemical studies in last four decades. The identification of two active site lysine residues 45-48 prompted the suggestion that reaction mechanism proceeds through formation of a Schiff's base (49). However, this idea was ruled out due to lack of experimental evidence (50).

Later, based on isotope exchange rates and spectroscopic studies, it was proposed that the reductive amination of α-ketoglutarate proceeds through an enzyme-bound α-iminoglutarate intermediate (13,19,50,51). The reaction mechanism involves the nucleophilic attack by ammonia on the α-ketoglutarate of GDH-NADPH-α-ketoglutarate ternary complex. The α-ketoglutarate carbonyl oxygen was proposed to be protonated by a catalytic lysine forming the carbaminolamine intermediate. Elimination of water from this carbaminolamine generates the α-iminoglutarate intermediate, which was spectroscopically identified in bovine GDH (17). Proton transfers in this step are possibly assisted by a carboxylate group of an aspartate (Asp165 in CsGDH) (13). The reduced coenzyme, stacking in close proximity to the newly formed imino group, completes the facile hydride transfer to form L-glutamate. Besides a catalytic carboxylate containing residue, two active site lysines (Lys113 and Lys125 in CsGDH) are also conserved in all NADP⁺-dependent GDHs. Despite numerous mechanistic studies, the direct structural evidences to capture the reaction intermediates was
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unavailable and the ammonia binding pocket was yet to be established.

The constellation of AnGDH active site residues and their binding mode with substrate, coenzyme, and reaction intermediates unraveled the snapshots of steps that occur during the catalytic reductive amination of α-ketoglutarate to L-glutamate. Our structural analysis reveals NADPH binding induces a major conformational change that places the substrate and coenzyme in a catalytically correct orientation. A water molecule forming hydrogen bond with Lys114 in AnGDH structures implies its catalytic importance. The ionic interactions of Asp154 with Arg82 and Lys114 suggests that two latter residues might play a crucial role in the catalysis. In a catalytically competent active site, the carboxylate group of Asp154 is likely to remain negatively charged by interacting with the positively charged side chains of Arg82 and Lys114 (Fig. 9). Our kinetics data on Arg82Gln AnGDH mutant and strict conservation of this residue (Fig. S2) among other GDHs implicate involvement of Arg82 in the catalytic reaction mechanism. The importance of Lys114 in catalysis was demonstrated before in CgGDH (30). The Asp154 side chain and the main chain of Gly153 might play a crucial role in anchoring an ammonium ion. The position of a water molecule, hydrogen bonded to these two groups in the AKG bound subunits of fiAnGDH structure (Fig. 5a) supports this view. Our structural analysis promotes the idea that the negatively charged carboxylate group of Asp154 and the polarized main chain carbonyl group of Gly153 are involved in positioning NH₄⁺ ion in the active site. The Asp154 carboxylate group deprotonates NH₄⁺ and facilitates nucleophilic attack by ammonia onto the α-carbon of α-ketoglutarate. The generated oxanion becomes protonated by the neighboring water molecule located near Lys114; this in turn regenerates water molecule by proton donation. A tetrahedral intermediate (2-amino-2-hydroxyglutarate) is formed in the active site and AnGDH-AIG-NADP⁺ structure directly supports formation of such an intermediate (Fig. 3b). Elimination of a water molecule leads to α-iminoglutarate formation. Subsequently, the hydride transfer from NADPH to α-iminoglutarate forms L-glutamate. The orientation of the nicotinamide ring of NADPH in the AnGDH-AKG-NADPH complex structure represents the catalytically competent conformation of the coenzyme capable of hydride transfer. Theoretical calculation on alcohol dehydrogenase reported (53) the required distance for hydride transfer between NAD⁺ and substrate to be around 2.7 Å; corresponding distances observed in the catalytically competent AnGDH structures are consistent. On the whole, collated structural data amply illuminate the key features of the GDH reaction mechanism.

Conclusions - Our results provide the structural basis of three important aspects related to catalysis by GDH: (a) cofactor specificity, (b) allosteric regulation, and (c) reaction mechanism. We have determined the first crystal structures of a fungal glutamate dehydrogenase. The complexed structures of AnGDH present direct evidences of formation of α-iminoglutarate and 2-amino-2-hydroxyglutarate as reaction intermediates. The different conformational states of AnGDH structures suggest that the allosteric regulation in this enzyme follows MWC model. The structural data reveal that the 2'-phosphate group of NADP(H) anchored to NADP(H)-GDH might have two possible ionization states. These findings resonate with other dehydrogenase mechanisms as well.

EXPERIMENTAL PROCEDURES

AnGDH expression and purification— The expression and purification of recombinant AnGDH was performed as described previously (5,26,27), with minor modifications. A single colony of E. coli BL21 (DE3) with AnGDH expression construct was grown overnight at 37 °C in LB broth medium with ampicillin (100 µg/ml). The culture (1% v/v) was re-inoculated into the LB medium containing ampicillin (100 µg/ml), grown at 37 °C until an optical density of 0.5 at 600 nm and then protein expression was induced.
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by addition of IPTG (400 µM). Protein expression was done by growing the culture at 22 °C for 12 h. The cell pellet obtained from 1 l culture was suspended in a lysis buffer (Buffer A: 100 mM potassium phosphate buffer pH 7.5, 1 mM EDTA and 1X protease inhibitor cocktail). After cell disruption using ultrasonication, the cell free extract was prepared by centrifugation at 12000 rpm and the supernatant was collected. Ammonium sulfate saturation was performed and the pellet obtained after 30-70 % saturation was dissolved in Buffer B (20 mM potassium phosphate buffer pH 7.5 and 1 mM EDTA). The sample was further desalted using Hiprep 26/10 desalting column. Next, the protein sample was loaded on to a 50 ml CR-12 dye affinity column (Novacron Red LS-BL coupled through an epoxy spacer arm to Sepharose) and elution was performed by a linear gradient of potassium chloride. The eluted sample was desalted and loaded on to a DEAE Sepharose (5 ml, HiTrap) column and bound proteins were eluted by a linear gradient of potassium chloride. The final purification of AnGDH was performed using a Superdex 200 16/60 gel filtration column. The purity of the protein was analyzed on SDS-PAGE (54). Protein concentrations were measured using Bradford’s method (55).

Enzyme assay – The enzyme activity of pure AnGDH was assayed as previously described (5). Reductive amination of α-ketoglutarate to L-glutamate is accompanied by oxidation of NADPH to NADP⁺. Therefore, the enzyme activity was measured by the initial rate of disappearance of NADPH absorbance at 340 nm. The change in absorbance (ΔA₃₄₀) was recorded per minute. For the forward reaction was carried out in 1 ml of total reaction mixture containing 100 mM Tris buffer pH 8.0, 10 mM α-ketoglutarate, 10 mM ammonium chloride and 0.1 mM NADPH. One enzyme activity unit refers to the amount of enzyme required to oxidize one µmol of NADPH per min under the standard assay conditions.

Site directed mutagenesis – PCR based site directed mutagenesis was performed to generate Ala-substituted AnGDH mutants at residues His84, Lys122, Ser253, Lys277 and Gln282 using pET43.1bAnGDH expression vector. Arg82Gln mutant was prepared in a similar way. The primers used for generating these mutants are listed in Table S1. Mutations were confirmed with DNA sequencing and the expression constructs of these mutants were transformed into ΔGDH E. coli BL21 (DE3) (56). Expression, purification and activity assays of the mutant enzymes were performed following the same procedures as described for recombinant wild type AnGDH. Correctness of folding of the mutants was confirmed by circular dichroism (CD) measurements.

Kinetics of wild type and mutant AnGDH - NADPH saturation of the wild type AnGDH and its mutant forms (His84Ala, Lys122Ala, Ser253Ala, Lys277Ala and Gln282Ala) was performed using reductive amination assay. This reaction was followed at pH 8.0 with varying substrate (NADPH) concentration. The standard assay (as mentioned in Enzyme assay section) was suitably modified. The substrate conversion was maintained below 10 % to achieve initial velocity. The NADPH concentration was varied while the enzyme and other substrate concentrations were kept constant. The saturation of NADH was performed in parallel.

The assay was suitably modified for the ammonia saturation kinetics of wild type AnGDH and its Arg82Gln mutant. NADPH and AKG concentrations were kept fixed, and ammonium chloride concentration was varied with suitable amount of the enzyme. All these experiments were performed in triplicates at room temperature.

Fluorescence quenching experiment – Purified AnGDH (1 mg/ml) in 20 mM phosphate buffer (pH 7.5) was used for measuring the substrate and cofactor induced fluorescence intensity change. All the experiments were performed in a fluorescence spectrophotometer (JASCO). For the tryptophan quenching in AnGDH, the excitation wavelength of 295 nm was used and the emission wavelength was in the range of 300-500 nm. The excitation and emission band widths were kept at 2.5 nm and slit width used was 2.5 nm. The reaction mixtures contained a fixed concentration (3 µM) of AnGDH and variable concentrations of α-ketoglutarate in a total reaction volume of 0.5 ml assembled in a quartz cuvette and a path length of 10 mm was used. Different concentrations of α-ketoglutarate used for the measurements were 2
mM, 5 mM, 7 mM, 9 mM, 15 mM and 20 mM. In separate experiment, fixed concentration (6 µM) of AnGDH was used with NADPH of varying concentrations (0.2 µM, 0.5 µM, 1 µM, 2 µM, 4 µM, 6 µM, 8 µM, 12 µM, 15 µM and 18 µM). The observed fluorescence intensities were corrected for the inner filter effect in the experiments. All the spectral measurements were carried out at 25 °C and in triplicates.

Preparation of forward inhibited AnGDH – The forward inhibited AnGDH (fiAnGDH) was prepared by treating the purified enzyme with 2 mM of 2-hydroxyethyl disulfide (2-HED) and incubating the mixture at 37 °C as described previously (27). Forward inhibition of the enzyme was confirmed by activity measurements as discussed in the previous section. The fiAnGDH preparation was further buffer exchanged with Buffer B, concentrated to 12 mg/ml and stored at 4 °C.

Crystallization – Active and forward inhibited forms of AnGDH were crystallized using sitting or hanging drop vapor diffusion method at 22 °C. Initial crystallization screens were set up using commercially available screen solutions: (a) JCSG Core-I suite (Qiagen), (b) PEGs suite (Qiagen) and (c) JCSG plus suite (Molecular Dimensions); with a Phoenix (Art Robins) crystallization robot available at the "Protein Crystallography Facility", Indian Institute of Technology Bombay. Crystallization trials for apo-AnGDH with a protein concentration of 7 mg/ml were set up using factorial 1 screening conditions (57). The first crystals of apo-AnGDH were obtained in a condition containing 15 % (w/v) PEG 3350, 0.1 M Tris-Cl and 0.2 M NaCl within one week. Further optimization of this condition using several additives produced the best quality apo-AnGDH crystals in a mother liquor having 20 % (w/v) PEG 3350, 0.1 M NaCl, 0.1 M Tris-Cl, pH 8.5 and 0.01 M BaCl2.

Crystallization of AnGDH complexed with α-ketoglutarate and NADPH (AnGDH-AKG-NADPH) was done as reported before (5). For complex formation, AnGDH (12 mg/ml) was mixed with α-ketoglutarate and NADPH for 30 min at 25 °C achieving 0.6 mM final concentrations of the substrate and coenzyme. The crystallization screens were set up and the initial crystals of the AnGDH-AKG-NADPH complex were observed within one week in a condition containing 0.1 M sodium citrate (pH 5.5) and 20 % (w/v) PEG 3000. These crystals grew to their maximum size within one week and were further used for diffraction studies.

In order to obtain crystals of AnGDH complexed with isophthalate and NADPH (AnGDH-IPT-NADPH), the concentrated (12 mg/ml) protein solution was mixed with these compounds and the mixture was incubated for 30 min at 25 °C. The final concentrations of isophthalate and NADPH in the mixture were of 0.8 mM and 0.6 mM, respectively. Crystallization screens of this complex were set up and the best crystals were obtained in a condition containing 0.1 M MES (pH 6.0) 30 % (v/v) PEG 200 and 5 % (w/v) PEG 3000. The crystals grew to their maximum size within two weeks.

Preparation of AnGDH complex with α-iminoglutarate (AnGDH-AIG-NADP+) was done by incubating the mixture of enzyme (12 mg/ml) with α-ketoglutarate, NADP+ and ammonium chloride at 25 °C for 5 min. The final mixture contained 1 mM α-ketoglutarate, 1 mM NADP+ and 0.5 M ammonium chloride. Crystallization screens of this complex were set up. After obtaining the first hit, the crystallization conditions were optimized and the best crystals were obtained in a condition containing 40 % (v/v) PEG 300, 0.1 M sodium cacodylate, pH 6.5 and 0.2 M calcium acetate hydrate. The crystals grew to their maximum size within two days.

The concentrated fiAnGDH (12 mg/ml) sample was incubated for 30 min at 25 °C with α-ketoglutarate and NADH to prepare a complex. The final concentrations of α-ketoglutarate and NADH used for preparing this complex were 0.2 M and 0.2 M, respectively. The crystallization screens for this complex were set up and the best crystals appeared after two weeks in a condition containing 0.15 M potassium bromide and 20 % (w/v) PEG 2000 MME, and grew to their maximum size in two weeks.

Data collection and processing – All the diffraction data were collected from the frozen crystals by the rotation method. The crystals were briefly transferred to their corresponding
cryoprotectant solutions using a nylon loop and subsequently flash-frozen in the liquid nitrogen stream at 100 K. A dataset for the apo-AnGDH crystal was collected using CuKα X-ray radiation source generated by a Bruker MICROSTAR diffractometer equipped with MAR345 detector at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India. The reservoir solution with 20 % (v/v) glycerol was used as a cryoprotectant for freezing the apo-AnGDH crystal. Diffraction data from fiAnGDH crystals were also collected using CuKα radiation generated by a Rigaku Micromax 007HF generator equipped with R-Axis IV++ detector at "Protein Crystallography Facility", IIT Bombay, India. The crystals of AnGDH-AKG-NADPH, AnGDH-IPT-NADPH and AnGDH-AIG-NADP+ complexes were first briefly transferred to the cryoprotectant solutions prepared from their respective mother liquors containing 30 % (v/v) glycerol, and then subsequently flash frozen in liquid nitrogen. The frozen crystals were then transferred to the liquid nitrogen stream at 100 K for data collection. The diffraction data sets from these complexes were collected at the BM14 beamline of European Synchrotron Radiation Facility (ESRF), Grenoble, France, using a MarCCD detector. Indexing, integration and scaling of all the data sets were performed by XDS (58). The intensities were converted to structure factors with the program modules F2MTZ and CAD of CCP4 (59). The data collection statistics are presented in Table 1.

Structure determination, model building and refinement – The structure of the apo-AnGDH was determined by molecular replacement. The A-subunit of E. coli glutamate dehydrogenase (EcGDH) crystal structure (PDB ID: 3SBO) which has an amino acid sequence identity of 55 % with AnGDH was used as the search model. Calculation of Matthews coefficient (2.7 Å³ Da⁻¹) (60) indicated the presence of six apo-AnGDH molecules in the asymmetric unit. The correct orientations of six subunits were identified by Phaser (61) using the search model. The hexameric unit of the model was refined for ten cycles using REFMAC5 (62). The resulting electron density map was used for automated model building using Buccaneer (63) which could correctly assign almost 60 % residues of the hexameric AnGDH structure. The partially built model was used for subsequent manual model building by visual inspection in COOT (64) and refinement using REFMAC5. The solvent molecules and ions were progressively added at peaks of electron density higher than 3σ in sigma-A weighted Fo-Fc electron density maps while monitoring the decrease of Rfree and improvement of the overall stereochemistry. In the structure, subunits A, B and C have one and subunits D, E and F have two N-terminal residues missing as they could not be built due to lack of features in the electron density.

The structures of AnGDH-AKG-NADPH complex and fiAnGDH were solved by molecular replacement using the coordinates of apo enzyme A-subunit. The initial phases of the structures of other AnGDH complexes were obtained by the rigid body refinement of protein part of the AnGDH-AKG-NADPH structure as all the complexed crystal forms had almost identical cell dimensions and belonged to the same space group. First few cycles of refinement of only the protein molecules were performed by REFMAC5. Subsequently, the ligands were placed inside the sigma-A weighted Fo-Fc electron density map and further refinement cycles were carried out. The waters and other solvent molecules were added to the structures, and alternate conformations of residues were built using COOT. Convergence of the refinement process was monitored by the decrease of Rfree and improvement of the overall stereochemistry. The refinement statistics of all the structures presented in this study are reported in Table 1.

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Table 1: Data collection and refinement statistics

| Data collection statistics                  | Apo-AnGDH | AnGDH-AKG-NADPH | fAnGDH-AKG | AnGDH-AIG-NADPH | AnGDH-IPT-NADPH |
|--------------------------------------------|-----------|-----------------|------------|-----------------|-----------------|
| Space group                                | P1        | P1              | P1         | P1              | P1              |
| Unit cell dimensions (Å)                   | 92.8, 92.8, 111.7 | 174.5, 174.5, 240.4 | 92.3, 92.2, 111.1 | 173.7, 173.7, 241.9 | 173.5, 173.5, 241.1 |
| α, β, γ (%)                                | 103.5, 94.2, 120.1 | 90.0, 90.0, 120.0 | 103.4, 93.5, 120.4 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| Resolution (Å)                             | 40.0-2.8 (2.9-2.8) | 35.0-1.8 (1.9-1.8) | 35.0-2.25 (2.35-2.25) | 35.0-1.75 (1.85-1.75) | 70.0-1.9 (2.0-1.9) |
| Wavelength (Å)                             | 1.5418 | 0.9763 | 1.5418 | 0.95372 | 0.9763 |
| Temperature (K)                            | 100 | 100 | 100 | 100 | 100 |
| Observed reflections                       | 1284717 (10559) | 1571927 (207073) | 270254 (31489) | 1103599 (168659) | 809288 (106968) |
| Unique reflections                         | 69072 (6502) | 128967 (18853) | 135737 (15991) | 138857 (21413) | 109208 (15446) |
| Rmerge (%)                                 | 92.0 (86.0) | 99.6 (97.9) | 95.5 (92.0) | 990.0 (99.9) | 999.0 (98.8) |
| Ramachandran plot allowed (%)              | 6.2 (28.6) | 9.2 (41.5) | 9.5 (39.8) | 10.9 (110.5) | 5.4 (41.9) |
| Ramachandran plot favoured (%)             | 8.8 (40.5) | 9.6 (43.5) | 13.4 (56.5) | 11.6 (118.3) | 5.8 (45.2) |
| For I                                      | 11.2 (3.6) | 17.1 (5.1) | 8.0 (2.1) | 13.4 (1.9) | 24.3 (4.5) |
| CC1-2 (%)                                  | 99.0 (83.7) | 99.8 (96.4) | 98.9 (67.9) | 99.8 (69.9) | 99.9 (61.3) |
| Redundancy                                 | 2.0 (1.6) | 12.2 (10.9) | 2.0 (1.9) | 7.9 (7.9) | 7.4 (6.9) |
| Number of reflections (working set/test set) | 65578/3451 | 122517/6448 | 128936/6786 | 131488/6920 | 103732/5460 |
| Number of reflections                     | 35.0-2.8 | 34.0-1.8 | 33.0-2.25 | 34.1-75 | 34-1.9 |
| Number of atoms                            | 20940 | 3669 | 20933 | 3544 | 3513 |
| Protein                                    | 601 | 674 | 1515 | 528 | 308 |
| Water                                      | 0 | 10 | 30 | 0 | 0 |
| AKG                                        | 0 | 48 | 0 | 0 | 48 |
| NADPH                                      | 0 | 0 | 0 | 0 | 0 |
| NADPH′                                     | 0 | 0 | 0 | 0 | 0 |
| β-mercaptoethanol (BME)                    | 0 | 0 | 24 | 0 | 0 |
| IPT                                        | 0 | 0 | 0 | 0 | 12 |
| AKI                                        | 0 | 0 | 0 | 10 | 0 |
| AHG                                        | 0 | 0 | 0 | 11 | 0 |
| Average isotropic B-factor (Å²) for active site liquids | 26.8 | 48.6 | - | - | - |
| AKG                                        | - | 24.0 | - | - | - |
| NADPH                                      | - | - | - | 22.4 | - |
| NADPH′                                     | - | - | - | - | 20.4 |
| IPT                                        | - | - | - | - | - |
| AKI                                        | - | - | - | 20.1 | - |
| AHG                                        | - | - | - | 31.6 | - |
| Average isotropic B-factor (Å²) of all atoms | 27.5 | 29.3 | 25.9 | 28.3 | 31.0 |
| Occupancy for active site liquids           | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| AKG                                        | - | - | - | - | - |
| NADPH                                      | - | 1.0 | - | - | - |
| NADPH′                                     | - | - | - | 1.0 | - |
| IPT                                        | - | - | - | - | 1.0 |
| AKI                                        | - | - | - | - | - |
| AHG                                        | - | - | - | 0.8 | - |
| R.M.S.D.                                    | 0.011 | 0.014 | 0.011 | 0.012 | 0.011 |
| Bond angle (°)                             | 1.42 | 1.65 | 1.43 | 1.65 | 1.44 |
| Protein geometry                           | 92.92 | 97.07 | 96.20 | 96.34 | 96.41 |
| Ramachandran plot allowed (%)              | 6.09 | 2.93 | 3.39 | 3.43 | 3.14 |
| Ramachandran plot outliers (%)             | 1.00 | 0.0 | 0.41 | 0.23 | 0.45 |
| PDB ID                                     | 5XV1 | 5XVX | 5XVV | 5XW1 | 5XW0 |

*Values in parentheses correspond to highest resolution shell.

Apo-AnGDH: AnGDH structure without any ligand in the active site, AnGDH-AKG-NADPH: AnGDH structure complexed with α-ketoglutarate (AKG) and NADPH, fAnGDH-AKG: forward inhibited AnGDH structure complexed with α-ketoglutarate (AKG), AnGDH-AIG-NADPH: AnGDH structure complexed with reaction intermediates α-iminoglutarate (AIG)/2-amino-2-hydroxyglutarate (AHG) and NADPH, AnGDH-IPT-NADPH: AnGDH structure complexed with inhibitor isophthalate (IPT) and NADPH.
Table 2: Active site cleft opening in different AnGDH structures

| Structure                      | Chain | Distances (Å) between Cα atoms of Arg280 and Lys122 | Position of subunits |
|--------------------------------|-------|----------------------------------------------------|----------------------|
| Apo AnGDH (hexamer)           | A     | 13.0                                               | Upper part of hexamer|
|                                | B     | 15.1                                               | Lower part of hexamer|
|                                | C     | 13.4                                               |                      |
|                                | D     | 19.7                                               |                      |
|                                | E     | 21.3                                               |                      |
|                                | F     | 19.4                                               |                      |
| AnGDH ternary complex (monomer)| A     | 6.0                                                |                      |
| fiAnGDH-AKG complex (hexamer) | A     | 12.2                                               | Upper part of hexamer|
|                                | B     | 14.8                                               |                      |
|                                | C     | 13.3                                               |                      |
|                                | D     | 20.2                                               |                      |
|                                | E     | 21.3                                               |                      |
|                                | F     | 19.2                                               |                      |

Table 3: Kinetic parameters for the NADPH saturation of the wild type AnGDH and its various mutants.

|                  | Wild type | His84Ala | Lys122Ala | Ser253Ala | Lys277Ala | Gln282Ala |
|------------------|-----------|----------|-----------|-----------|-----------|-----------|
| NADPH            |           |          |           |           |           |           |
| \( K_m \)        | 24 ± 2.1  | 25.6 ± 2.2| 53.8 ± 0.9| 44.8 ± 0.7| 132.4 ± 1.2| 66 ± 1.3  |
| \( k_{cat} \)    | 198.3 ± 1.3| 123.7 ± 0.8| 145.6 ± 0.4| 1.7 ± 0.3 | 28.3 ± 0.8 | 0.17 ± 0.03|
| \( k_{cat}/K_m \)| 8.3 ± 0.8 | 4.8 ± 0.4 | 2.7 ± 0.05| 3.7×10^{-2}± 0.005| 2.1×10^{-1}± 0.005| 2.5×10^{-3}± 4×10^{-4}|

Units of \( K_m \) for NADPH and \( k_{cat} \) are \( \mu M \) and \( s^{-1} \), respectively. \(^a\) The \( S_{0.5} \) value is reported. The data is represented with the standard error of the mean.
FIGURE LEGENDS

FIGURE 1: Structural fold and flexibility of AnGDH. (a) Tertiary structure of AnGDH is shown as cartoon. The helices (cylinder) are marked with “H” and the strands (arrows) are marked with “β”. The star at the centre of two domains represents substrate binding site. (b) The topology diagram showing the arrangement of secondary structural elements. (c) Hexameric assembly shown in cartoon representation with each subunit with different color. The structure is viewed from a direction perpendicular to the 3-fold axis (arrow). (d) Conformational variabilities of domain II among the subunits in AnGDH hexamer. (e) Superposition of open (purple) and super-closed (cyan) conformations of AnGDH structures showing the domain closure and structural flexibility. Bound α-ketoglutarate and NADPH are shown as sticks. The positions of the Cα-atoms of Lys122 and Arg280 are shown as spheres. (f) Schematic diagram depicting conformational change in AnGDH structure upon substrate and coenzyme binding.

FIGURE 2: Active site of AnGDH complexed with α-ketoglutarate (AKG) and NADPH. (a) The sigma-A weighted F_o-F_c omit electron density maps of AKG (light brown carbon) and NADPH (yellow carbon) contoured at 3.0 σ level, with the final refined models superimposed. The inset shows the tetrahedral geometry of C4 atom in the nicotinamide ring of NADPH and its close proximity for hydride transfer to the α-carbon of AKG. Covalent structures of AKG and reduced form of nicotinamide ring are also shown. (b) Binding pocket for AKG in the active site of AnGDH. The residues are shown as light grey colored carbon. Polar interactions are shown as dotted lines. Water molecules are shown as spheres. (c) NADPH binding pocket in the AnGDH active site. Representation style is same as in panel b.

FIGURE 3: Active site of AnGDH complexed with α-iminoglutarate (AIG), 2-amino-2-hydroxyglutarate (AHG), and NADP⁺. (a) The reaction steps in AnGDH active site leading to the formation of AHG and AIG. (b) The electron density map guided refinement process of the reaction intermediates in the active site. (i) Initial sigma-A weighted F_o-F_c omit electron density map (green) contoured at 3.0 σ level showing predominant features of AIG and NADP⁺. (ii) The positive F_o-F_c omit map (green) contoured at 3.0 σ level indicates partial occupancy of AHG. (iii) Refined 2F_o-F_c map (blue) contoured at 1.0 σ level, after refinement of AIG, AHG and NADP⁺. (c) The sigma-A weighted F_o-F_c omit electron density maps contoured at 3.0 σ level, with the final refined models AIG (light magenta carbon), AHG (light brown carbon) and NADP⁺ (grey carbon) superimposed. (d) Binding pocket for AIG and AHG in the active site of AnGDH. The residues are shown as light grey colored carbon. Polar interactions are shown as dotted lines.

FIGURE 4: Active site of AnGDH complexed with isophthalate (IPT) and NADPH. (a) The sigma-A weighted F_o-F_c omit electron density maps of IPT (grey carbon) and NADPH (yellow carbon) contoured at 3.0 σ level, with the final refined models superimposed. (b) Comparison of mode of binding of AKG (cyan) and isophthalate (green).

FIGURE 5: Conformation flexibility of α-ketoglutarate (AKG) in the active site of AnGDH. (a) The binding mode of AKG (light brown carbon) in the active site of fiAnGDH (grey carbon). The sigma-A weighted F_o-F_c omit electron density map contoured at 3.0 σ level is also shown as green mesh around the final refined model of AKG and water molecule is presented as sphere. (b) Conformational differences of AKG in the coenzyme bound ternary (blue carbon) and unbound binary (brown carbon) AnGDH complexes.

FIGURE 6: Interactions among the hexamers of fiAnGDH-AKG complex in the crystal. (a) AKG binding to AnGDH (3 μM) active site shows cooperativity. (b) NADPH binding to AnGDH (6 μM). Insets in (a) and (b) panels show the change in fluorescence intensities (colored lines) upon addition of substrate (AKG) and coenzyme (NADPH), respectively to the AnGDH in one of the experiments. (c) Interactions
among the hexamers of fiAnGDH-AKG complex in the crystal. Each subunit of the hexamers is shown in different color (A: green, B: cyan, C: yellow, D: magenta, E: grey, F: blue). The lateral and vertical arrangements of the hexamers in the crystal. (d) Symmetric lateral organization of the hexamers in the crystal, viewed from the top. (e) The zoomed in view of the lateral interactions present in one side of the hexamer. (f) Vertical arrangement of the hexamers. (g) Representation of the interactions present in the vertical orientation.

FIGURE 7: Structural basis of AKG homotropic cooperativity in AnGDH. Schematic model representing the cooperativity in AnGDH.

FIGURE 8: The structural basis of cofactor selectivity in AnGDH. (a) Stereo view showing the interactions responsible for recognition of NADPH by AnGDH. The residues are shown in light brown colored carbon. Part of the bound NADPH is shown with grey color carbon. The polar interactions are shown by dotted lines. The final $2F_o-F_c$ electron density map contoured at 1σ level is also shown as purple colored mesh. (b) Schematic representation of different ionization states of the 2'-phosphate group of NADPH in the AnGDH active site. The red sphere on the right panel represents the oxygen atom of water. (c) The segment of polypeptide responsible for coenzyme recognition in NADP⁺-dependent GDHs. The GDH sequences from A. niger (An), Escherichia coli (Ec), Agaricus bisporus (Ab), Streptococcus suis (Ss), Salmonella enteric (Se), Corynebacterium glutamicum (Cg), Pseudomonas aeruginosa (Pa), Penicillium chrysogenum (Pc), Methylobacillus flagellates (Mf), Mycobacterium smegmatis (Ms), and Saccharomyces cerevisiae (Sc) have been used for the alignment. The secondary structural elements and residue numbers of AnGDH are shown on the top of the alignment. Ten conserved residues in Rossmann fold essential for 2'-phosphate group recognition in NADP⁺-dependent GDHs are numbered at the bottom of the alignment. The strictly conserved residues are shown as white on the red color background. (d) Secondary structural motif (with ten residues as stick) involved in NADPH recognition in AnGDH.

FIGURE 9: Reaction mechanism for reductive amination of α-ketoglutarate by AnGDH as supported by the structural data.
Figure 1
Structural insights into the catalytic properties of GDH

Figure 2
Figure 3
Structural insights into the catalytic properties of GDH

Figure 4
Figure 6
Figure 7
Structural insights into the catalytic properties of GDH

Figure 8
Structural insights into the catalytic properties of GDH

Figure 9
Structural basis for the catalytic mechanism and α-ketoglutarate cooperativity of glutamate dehydrogenase
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