Structural Basis for Leucine-induced Allosteric Activation of Glutamate Dehydrogenase*\[5\]

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Background: Glutamate dehydrogenase (GDH) from *Thermus thermophilus*, which plays an important role in the interconnection of nitrogen and carbon metabolism, is activated by leucine like mammalian GDH.

Results: The crystal structure of GDH from *T. thermophilus* was determined in a leucine-bound form.

Conclusion: The activation is caused by leucine binding at subunit interfaces.

Significance: The allosteric mechanism is suggested to be common between mammalian and *T. thermophilus* GDHs.

Glutamate dehydrogenase (GDH) catalyzes the reversible conversion between glutamate and 2-oxoglutarate using NAD(P)(H) as a coenzyme. Although mammalian GDH is regulated by GTP through the antenna domain, little is known about the mechanism of allosteric activation by leucine. An extremely thermophilic bacterium, *Thermus thermophilus*, possesses GDH with a unique subunit configuration composed of two different subunits, GdhA (regulatory subunit) and GdhB (catalytic subunit). *T. thermophilus* GDH is unique in that the enzyme is subject to allosteric activation by leucine. To elucidate the structural basis for leucine-induced allosteric activation of GDH, we determined the crystal structures of the GdhB-Glu and GdhA-GdhB-Leu complexes at 2.1 and 2.6 Å resolution, respectively. The GdhB-Glu complex is a hexamer that binds 12 glutamate molecules: six molecules are bound at the substrate-binding sites, and the remaining six are bound at subunit interfaces, each composed of three subunits. The GdhA-GdhB-Leu complex is crystallized as a heterohexamer composed of four GdhA subunits and two GdhB subunits. In this complex, six leucine molecules are bound at subunit interfaces identified as glutamate-binding sites in the GdhB-Glu complex. Consistent with the structure, replacement of the amino acid residues of *T. thermophilus* GDH responsible for leucine binding made *T. thermophilus* GDH insensitive to leucine. Equivalent amino acid replacement caused a similar loss of sensitivity to leucine in human GDH2, suggesting that human GDH2 also uses the same allosteric site for regulation by leucine.

Glutamate dehydrogenase (GDH)* catalyzes the reversible conversion between glutamate and 2-oxoglutarate using NAD(P)(H) as a coenzyme (Fig. 1). Because of its important role in the interconnection of nitrogen and carbon metabolism in cells, GDH is widely distributed among living organisms (1). The GDH50 enzymes (the most widely distributed family of GDH composed of six subunits, each with a molecular mass of ~50 kDa) are classified into two subtypes: mammalian GDH with an ~50-amino acid insertion that forms an antenna responsible for heterotropic control by GTP and GDH lacking the antenna sequence. Mammalian GDH is regulated in a complicated manner (2). ADP, NAD+, and leucine activate mammalian GDH, whereas ATP, GTP, NADH, and palmitoyl-CoA act as allosteric inhibitors. Mutations conferring resistance to GTP inhibition cause hyperinsulinism/hyperammonemia syndrome in humans by increasing the ratio of ATP to ADP through activation of the TCA cycle by providing 2-oxoglutarate, which in turn results in the stimulation of insulin secretion through closure of the ATP-sensitive potassium channel and depolarization of β-cells to open a voltage-gated calcium channel for calcium influx (3–5). Thus, GDH is thought to be a target for the development of therapeutic medicine for hyperinsulinism/hyperammonemia disease. In hyperinsulinism/hyperammonemia patients with GDH mutations, insulin secretion is hyperresponsive to leucine stimulation because GDH mutants are not inhibited by elevated levels of GTP (6); therefore, it is presumed that leucine-induced activation of GDH occurs in a manner independent of that in the GTP-mediated inhibitory mechanism. Although the hyperresponsiveness of insulin secretion to leucine stimulation in hyperinsulinism/hyperammonemia patients with GDH mutations is suppressed by glucose addition, desensitization to leucine stimulation by modulating GDH function would be desirable; however, the mechanism of leucine-mediated allosteric activation of mammalian GDH has not yet been elucidated.

Although microbial GDH lacks the antenna, it has also been characterized as regulated. Fungal GDHs from *Neurospora crassa* and Aspergillus species are regulated in a homotropic manner by glutamate (7, 8). *N. crassa* GDH is also activated by non-substrate di- or polycarboxylic acids, suggesting heterotropic regulation in this GDH. As for bacterial GDH, GDH from *Clostridium symbiosum* has been shown to be regulated in a homotropic manner (9). Recently, another type of GDH,
GDH180, which is a very large GDH with N- and C-terminal extension domains, has been reported to be regulated heterotropically by amino acids: GDHs from *Streptomyces clavuligerus* (activated by aspartate and asparagine) (10), *Janthinobacterium lividum* (activated by aspartate and arginine) (11), and *Pseudomonas aeruginosa* (activated by arginine and inhibited by citrate) (12). Thus, allosteric regulation by amino acids suggests that GDH plays important roles in metabolic control at the linkage of carbon and nitrogen metabolism in various organisms.

Recently, we found that *Thermus thermophilus* possesses two genes, both encoding glutamate dehydrogenase homologs on the genome, and these two homologs form a heterohexamer, which is allosterically activated by hydrophobic amino acids, especially leucine (13). In the heterocomplex, GdhB act as a catalytic subunit, whereas GdhA is cata
l
tically inactive and serves as a regulatory subunit. The leucine-induced activation is prominent in the form of the heterocomplex. Here, we determined the crystal structures of the GdhB-Glu and GdhA-GdhB-Leu complexes and found a novel allosteric site located at the interfaces of subunits in the hexameric structure. On the basis of the first crystal structures of hetero-oligomeric GDH from *T. thermophilus* and comparison with the primary and three-dimensional structures of mammalian GDHs, we provide here a structural basis for leucine-mediated activation of GDHs and also provide evidence that human GDH uses the same allosteric site for leucine activation.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Chemicals—**E. coli BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA) was used as the host to express *gdh* genes for *T. thermophilus* and the *GLUD2* gene encoding human GDH2 (hGDH2). 2-mercaptoethanol was added to the medium when 95% purity by SDS-PAGE. Crystallization was performed using the hanging drop vapor diffusion method using 0.1 M HEPES-NaOH (pH 7.0) and 0.6 M ammonium phosphate as the reservoir solution. The sample was equilibrated at 293 K, and crystals were obtained in a few days.

For crystallization of the GdhA-GdhB-Leu complex, we prepared GdhA-GdhB, both without tags. The gene encoding GdgA was amplified with a pair of primers, GdhA-Fw and GdhA-Rv, whereas the gene encoding GdhB was amplified with primers GdhB-Fw and GdhB-Rv (supplemental Table S1). The amplified DNA fragments for the *gdhA* and *gdhB* genes were cloned into the expression vector pET-26b(+) in this order to yield pET-GdhA-GdhB. E. coli cells harboring pET-GdhA-GdhB were grown in 2 × yeast extract/Tryptone medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) at 310 K. When the absorbance of the culture at 600 nm reached ~0.6, gene expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside, and culture was continued for an additional 12 h at 298 K. The cells were harvested, washed, and suspended in buffer A (20 mM Tris-HCl (pH 8.0) and 10 mM leucine). Suspended cells were disrupted by sonication and centrifuged at 40,000 × g for 15 min. The supernatant was heated at 353 K for 20 min to denature proteins from *E. coli*. The heated solution was centrifuged at 40,000 × g for 15 min, and ammonium sulfate was added to the supernatant at 70% saturation to precipitate the GdhA-GdhB complex. After centrifugation at 40,000 × g for 15 min, the precipitant was dissolved in buffer A. The sample was applied to a HiLoad 26/10 phenyl-Sepharose hydrophobic interaction column (GE Healthcare) equilibrated with buffer A supplemented with 1.5 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (1.5 to 0 M). The collected sample was concentrated and applied to a RESOURCE Q anion exchange column (6 ml, GE Healthcare) equilibrated with buffer A and eluted with a linear gradient of NaCl (0–1 M). The sample was pooled, concentrated, and applied to a HiLoad 26/60 Superdex 200 gel filtration column equilibrated with buffer A supplemented with 150 mM NaCl. Fractions were pooled and concentrated by Vivaspin 20 centrifugal filtration with a 10-kDa cutoff (Vivasience, Göttingen, Germany). GdhB thus prepared was confirmed to have >95% purity by SDS-PAGE. After the addition of glutamate at 100 mM, the GdhB concentration was adjusted to 5 mg/ml. Crystallization was performed by the hanging drop vapor diffusion method using 0.1 M HEPES-NaOH (pH 7.0) and 0.6 M ammonium phosphate as the reservoir solution. The sample was equilibrated at 293 K, and crystals were obtained in a few days.

**Crystallization of GDH-Leucine Complex**

For crystallization of the GdhA-GdhB-Leu complex, GdhA-GdhB was grown in 2 × yeast extract/Tryptone medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) at 310 K. When the absorbance of the culture at 600 nm reached ~0.6, gene expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside, and culture was continued for an additional 12 h at 298 K. The cells were harvested, washed, and suspended in buffer A (20 mM Tris-HCl (pH 8.0) and 10 mM leucine). Suspended cells were disrupted by sonication and centrifuged at 40,000 × g for 15 min. The supernatant was heated at 353 K for 20 min to denature proteins from *E. coli*. The heated solution was centrifuged at 40,000 × g for 15 min, and ammonium sulfate was added to the supernatant at 70% saturation to precipitate the GdhA-GdhB complex. After centrifugation at 40,000 × g for 15 min, the precipitant was dissolved in buffer A. The sample was applied to a HiLoad 26/10 phenyl-Sepharose hydrophobic interaction column (GE Healthcare) equilibrated with buffer A supplemented with 1.5 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (1.5 to 0 M). The collected sample was concentrated and applied to a RESOURCE Q anion exchange column (6 ml, GE Healthcare) equilibrated with buffer A and eluted with a linear gradient of NaCl (0–1 M). The sample was pooled, concentrated, and applied to a HiLoad 26/60 Superdex 200 gel filtration column equilibrated with buffer A supplemented with 150 mM NaCl. Fractions were pooled and concentrated to 10 mg/ml. The GdhA-GdhB complex was confirmed to have >95% purity by SDS-PAGE. Crystallization was performed by the hanging drop vapor diffusion method using 0.1 M sodium citrate tribasic (pH 5.6) and 0.2 M ammonium phosphate monobasic as the reservoir solution. The sample was equilibrated at 293 K, and crystals were obtained in a few days.

**X-ray Data Collection, Structure Determination, and Refinement—**Prior to data collection of the GdhB-Glu and GdhA-GdhB-Leu complexes, crystals were briefly soaked in reservoir solution supplemented with 25% (v/v) glycerol as a cryoprotectant and flash-cooled in a nitrogen gas stream at 95 K. The diffraction data of the GdhB-Glu and GdhA-GdhB-Leu complexes were collected at the NE-3A and NW-12 stations, respectively, of the Photon Factory of the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan). Diffraction images were indexed, integrated, and scaled using the
Crystal Structure of GDH-Leucine Complex

HKL2000 program suite (14). The structure of the GdhB-Glu complex was determined by molecular replacement with the program Phaser (15) in the CCP4 program suite (16) using the structure of GDH from *Thermotoga maritima* as the search model (Protein Data Bank code 1B26) (17). The structure of the GdhA-GdhB-Leu complex was determined by molecular replacement using the structure of the GdhB-Glu complex as the search model. Refinement and model correction in the electron density maps were performed with Refmac 5.5 (18) and Coot (19). Data collection, refinement statistics, and the results of Ramachandran plots produced by the program PROCHECK (20) for both structures are summarized in *supplemental Table S2*. Three-dimensional structures were drawn using PyMOL. The atomic coordinates and structure factors for the GdhB-Glu (code 3AOG) and GdhA-GdhB-Leu (code 3AOE) complexes have been deposited in the Protein Data Bank.

Preparation of *T. thermophilus* GDH Mutants from *E. coli*—To prepare site-directed mutants, appropriate mutations were introduced into the *gdhA* gene on the pBluescript II SK(+) vector using a QuikChange kit (Stratagene) and the oligonucleotide primers listed in *supplemental Table S1*. The *gdhA* gene with the desired mutation was coexpressed with the wild-type *gdhB* gene in *E. coli* using two compatible expression vectors pETDuet-1 and pACYCDuet-1 (Novagen), and the heterocomplex was purified as described previously (13). Proteins were determined by the Bradford method using a Bio-Rad protein assay kit.

Enzyme Assay of GDH Activity—To measure the GDH activity of the GdhA-GdhB heterocomplex and mutants, the reaction mixture for reductive amination of 2-oxoglutarate contained 100 mM potassium phosphate buffer (pH 7.0), 50 mM NH₄Cl, 50 mM 2-oxoglutarate, and 150 μM NADH. The reaction was started by the addition of an appropriate amount of enzymes to the reaction mixture preheated at 333 K, and the oxidation of NADH to NAD⁺ was monitored at 340 nm in a Shimadzu UV2000 spectrophotometer. The activity of hGDH2 was measured at 298 K in a similar manner with a minor modification: the 2-oxoglutarate concentration was set at 10 mM, and 200 μM NADPH was used. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of NAD(P)H/min. To examine the response to leucine of the mutant GdhA-GdhB complex and hGDH2, leucine was added to the assay buffer at 0, 1, 10, 100, and 1000 μM. To examine the response to leucine of hGDH2, 10,000 μM leucine was also used.

Preparation of Recombinant hGDH2 and Mutants—To construct the plasmid for the expression of the GLUID2 gene encoding hGDH2, the DNA fragment was amplified from the cDNA prepared from human brain mRNA (kindly provided by Dr. C. Nishiyama, Juntendo University School of Medicine) by PCR with a pair of synthetic oligonucleotides, hGDH2-N and hGDH2-C (see *supplemental Table S1*). The amplified fragments were digested with BamHI/EcoRI and cloned into pBluescript II SK(+). After the nucleotide sequence was verified, the DNA fragment was cloned into multiple cloning sites of pHIS8 (21). The resulting plasmid, pHIS8-hGDH2, was designed to direct the expression of mature hGDH2 lacking the 53 amino acid residues of the N-terminal mitochondrion-targeting sequence in a form with an N-terminal extension of the 22 amino acid residues containing the His₈ tag derived from the vector. For overexpression of hGDH2, *E. coli* cells harboring pHIS8-hGDH2 were cultured in 2× yeast extract/Tryptone medium supplemented with 50 μg/ml kanamycin and 30 μg/ml chloramphenicol. When *E. coli* cells were grown to an absorbance of ~0.6 at 600 nm, isopropyl β-D-thiogalactopyranoside was added at a final concentration of 0.1 mM. The culture was continued for an additional 12 h after induction at 298 K. *E. coli* cells from 1.6 liters of culture were suspended in 32 ml of buffer B (20 mM Tris-HCl (pH 8.0) and 10% (v/v) glycerol) and disrupted by sonication. The supernatant prepared by centrifugation at 40,000 × g for 30 min was applied to a nickel-nitriolitrate resin column (Novagen) pre-equilibrated with buffer B containing 20 mM imidazole. After washing the column with a sufficient volume of buffer B containing 20 mM imidazole, hGDH2 was eluted with buffer B containing 500 mM imidazole. The purity of the recombinant enzyme was verified by 12% SDS-PAGE. The recovered fraction was concentrated with a Vivaspin 20 concentrator. To construct site-directed mutants, appropriate mutations were introduced into pHIS8-hGDH2 using the oligonucleotide primers listed in *supplemental Table S1*. The mutant enzymes were purified in the same way as described for wild-type hGDH2.

RESULTS AND DISCUSSION

Homohexameric Structure of the GdhB-Glu Complex—The structure of the GdhB-Glu complex was determined at 2.1 Å resolution by the molecular replacement method using the structure of GDH from *T. maritima* as the search model. GdhB has a homohexameric structure similar to other bacterial and mammalian GDH50 enzymes (Fig. 2A) (17, 22–26). A hexamer binds 12 glutamate molecules; six molecules are bound at the active sites, whereas the other six are bound at the subunit interfaces. Glutamate molecules bound to the active site are stabilized by specific interaction (Fig. 2B). The α-amino and the α-carboxyl groups of glutamate electrostatically interact with Asp-154 and Lys-114, respectively. The γ-carboxyl group of glutamate electrostatically interacts with Arg-194 and forms a hydrogen bond with Ser-357. In GDH from *C. symbiosum*, it has been suggested that the conserved Asp-165 residue corresponding to Asp-154 in GdhB plays a crucial role in accepting a proton from the α-amino group of glutamate in oxidative deamination (25), and the conserved Lys-125 corresponding to Lys-114 in GdhB polarizes a bound water molecule to attach the iminoglutamate intermediate (Fig. 2C) (25). In GdhB, because glutamate is bound to the active site of GdhB in a manner roughly similar to that in the glutamate complex of GDH from *C. symbiosum* (25), we can conclude that these residues play the same role in the catalytic function in GdhB.

In GDH from *C. symbiosum*, Lys-113 forms a hydrogen bond with Asn-373 from the NAD⁺ binding domain in the apo-form with an open conformation (25). Upon glutamate binding, the side chain conformation of Lys-113 is altered to interact with the α-carboxyl group of the bound glutamate with the hydrogen bond to Asn-373 maintained, which causes the enzyme to take a closed conformation. In the GdhB-Glu complex, Lys-102 corresponding to Lys-113 in GDH from *C. symbiosum* is hydrogen-bonded to Asn-350 corresponding to Asn-373 in GDH.
from *C. symbiosum*; however, it does not form a hydrogen bond with the $\alpha$-carboxyl group of the bound glutamate molecule. Thus, the GdhB-Glu complex takes an open-like structure, although it binds the glutamate molecule at the active site. The loss of the hydrogen bond between Lys-102 and the $\alpha$-carboxyl group of the bound glutamate in the GdhB-Glu complex suggests that the closed complex is not fully stabilized in the homo-hexamer composed of only GdhB subunits. GdhB shows activity in both reductive amination and oxidative deamination for 2-oxoglutarate and glutamate, respectively; however, the activity is 4–7-fold lower than that of the GdhA-GdhB heterohexamer in the presence of leucine (13). We assume that the determined GdhB structure may represent a low-activity state not activated by leucine. The carboxyl oxygen atom of the bound glutamate occupies the position of the catalytic water molecule in GDH from *C. symbiosum*. Upon leucine binding, the carboxyl side chain of the bound glutamate may change the direction to interact with Lys-102, and a water molecule may occupy the vacant space.

Six other glutamate molecules are bound at the interfaces of three subunits (Fig. 2D); for example, the $\alpha$-amino and $\alpha$-carboxyl groups of the bound glutamate are recognized by Asp-167 from the E chain and Arg-135 from the B chain, respectively. The amide and carbonyl groups of the Tyr-423 main chain from the A chain interact with the $\alpha$-carboxyl and $\alpha$-amino groups of the bound glutamate, respectively. The bound glutamate extends its carboxyl side chain toward Thr-72 and Ala-73 from the A chain, but the small side chains of Thr-72 and Ala-73 allow the side chain of the bound glutamate to fit in the pocket. The carboxyl side chain is stabilized by weak electrostatic interaction (distance of 3.5 Å) with Arg-420 from the A chain.

**Heterohexameric Structure of the GdhA-GdhB-Leu Complex**—The structure of the GdhA-GdhB-Leu complex was determined at 2.6 Å resolution. GdhA-GdhB forms a heterohexameric structure, which is composed of four GdhA subunits and two GdhB subunits (Fig. 3A). Here, we named each subunit GdhBA, GdhBB, GdhAC, GdhAD, GdhAE, and GdhAF. Native PAGE indicated that the GdhA-GdhB preparation coexpressed in *E. coli* was a mixture of GdhA$_2$-GdhB$_4$ and GdhA$_4$-GdhB$_2$ heterohexamers as the major components (13). Because it is very difficult to separate these two components due to their similar molecular behaviors, we performed crystallization using the mixture. As a result, the GdhA$_4$-GdhB$_2$ component was crystallized under the conditions described under “Experimental Procedures.” The overall structure suggests that two trimers, each forming a ring, are stacked together, base to base; however, in the heterohexamer, three dimers composed of two
identical subunits, each from the upper and bottom rings, associate to form a hexamer. In the heterohexameric structure, the four GdhA subunits form two dimers, and the two GdhB subunits form a dimer. In a dimer, one GdhA(B) subunit from the upper ring is related to the other GdhA(B) subunit from the bottom ring by 2-fold symmetry. The heterohexameric structure is formed by the association of three dimers: two GdhA dimers and one GdhB dimer in pseudo-3-fold symmetry (Fig. 3B). This subunit arrangement is explained by differences in the conservation of amino acid residues at subunit interfaces. When the amino acid residues of GdhA forming the GdhA/GdhA dimer interface are compared with those of GdhB forming the GdhB/GdhB dimer interface, only 46% of the amino acid residues are conserved between GdhA and GdhB (supplemental Fig. S1). In addition, in the corresponding interfaces, helices containing Glu-38 and Ser-39 are dislocated, and the N-terminal loop regions containing Ser-7–Leu-9 take different conformations between the GdhA dimer and the GdhB dimer (supplemental Fig. S2, A–E). These structural differences prevent the formation of the heterodimer, and only homo-assembled dimerization is allowed in the heterocomplex. In contrast, the amino acid residues in the interfaces related by the pseudo-3-fold symmetry are well conserved (~75% identities) (supplemental Fig. S1) and occupy similar positions between GdhA and GdhB (supplemental Fig. S3, A and B). These structural similarities between GdhA and GdhB in the interfaces related by pseudo-3-fold symmetry enable the hetero-association between GdhA dimers and the GdhB dimer using the interfaces.

The domain opening/closing cycle between the catalytic and nucleotide-binding domains is known to be important in the catalytic mechanism of GDH (27). In the GdhA-GdhB-Leu complex, the GdhA subunit takes a closed-like structure similar to the form of bovine GDH complexed with GTP (28), whereas the GdhB subunit that does not bind glutamate takes an open-like structure with an ~12° opening of the nucleotide-binding domain compared with the GdhA subunit (Fig. 3C).

Although no substrate is found in the active site of the GdhA-GdhB-Leu complex, six leucine molecules are found at the interfaces of three subunits (Fig. 3, A and B). These six leucine-binding sites can be classified into three types (sites 1–3) based on the difference in subunit organization. Site 1 is formed by three GdhA subunits, site 2 is formed by two GdhA subunits and one GdhB subunit, and site 3 is formed by one GdhA subunit and two GdhB subunits. The GdhA4-GdhB2 heterohexamer contains two for each site. The amino acid residues forming site 1 are identical to those forming site 2, and leucine is recognized in the same way at both sites; therefore, we describe only sites 1 and 3 (Fig. 3, D and E). At site 1 formed by GdhA4, GdhA5, and GdhA6, for example, the α-carboxyl group of the bound leucine forms bidentate ionic pairs with Arg-134 from the GdhA5 subunit and forms hydrogen bonds and electrostatic interactions indicated by dashed lines. The Fo − Fc map of Leu and the Leu-binding site at Leu site 3 contoured at 3.2σ is shown as black mesh.
α-amino group of the bound leucine forms an electrostatic interaction with Asp-166 from the GdhA4 subunit and forms a hydrogen bond with the main chain carbonyl group of Tyr-418 from the GdhA4 subunit (Fig. 3D). The side chain of leucine is stabilized by hydrophobic interaction with Ile-71 and Ala-72 from the GdhA4 subunit, and the aromatic side chain of Tyr-38 from the GdhA4 subunit and the hydrophobic side chain of Met-170 from the GdhA4 subunit further stabilize leucine binding. At site 3 formed by GdhBA, GdhBB, and GdhAE, for example, recognition of leucine was somewhat weaker than at sites 1 and 2 (Fig. 3E). Tyr-38 is replaced with Ser-39 at site 3. The replacement must cause loss of a hydrogen bond with the carboxyl group of the bound leucine and hydrophobic interaction with the side chain of leucine at site 3. Replacement of Ile-71 with Thr-72 may reduce hydrophobic contact with the side chain of leucine at site 3 compared with those at sites 1 and 2. These differences possibly provide site 1 (site 2) and site 3 with different sensitivity to leucine; the former is a high-affinity site, and the latter is a low-affinity site. In our previous study, we showed that glutamate exhibits negative cooperativity on allosteric regulation (13). Because the GdhA-GdhB preparation used in our previous study was a mixture containing GdhA4-GdhB2 and GdhA2-GdhB4 as major components, we cannot interpret the observed negative cooperativity of glutamate precisely from the crystal structure of GdhA4-GdhB2. In the present study, however, we found that leucine-binding sites 1–3 have the potential to be used for binding glutamate, as shown in the GdhB-Glu complex. Therefore, we assume that leucine-binding sites with different structures may accept glutamate with different affinity, which may account for the negative cooperativity by glutamate in the GdhA-GdhB complex.

**Leucine Binding Is Responsible for Allosteric Activation**—In our previous study, we showed that leucine affects the GDH activity of the GdhA-GdhB heterocomplex more than that of GdhB (13). These observations suggest that the GdhA subunit possesses crucial residues that determine the sensitivity to leucine. To verify whether leucine bound to the subunit interfaces in the crystal structure is actually involved in the allosteric activation of GDH, we constructed and characterized mutant GDHs, each with a single amino acid replacement at the leucine-binding sites of the GdhA subunits. The wild-type GdhA-GdhB heterocomplex increased the specific activity for reductive amination of 2-oxoglutarate as the leucine concentration increased (Fig. 4A). As expected, all of the mutants, Y38S, I71T, A72D, R134A, and D166A, which are involved in leucine binding in the crystal structure, exhibited markedly decreased sensitivity to leucine (Fig. 4, B–F). These results demonstrate that these residues play a major role in binding leucine for allosteric activation and that allosteric activation is caused by binding leucine at subunit interfaces. It should be noted that the mutations also affected the specific activity of these mutants: 8.71, 1.83, 2.24, 1.45, 3.40, and 7.11 units/mg for wild-type GdhA-GdhB, GdhA(Y38S)-GdhB, GdhA(I71T)-GdhB, GdhA(A72D)-GdhB, GdhA(R134A)-GdhB, and GdhA(D166A)-GdhB, respectively. The substantial activities of these mutants indicate that these residues are not directly involved in the catalytic mechanism; however, the obvious changes in the specific activities of the mutants at the subunit interface suggest that the subunit interaction may affect the catalytic events, as discussed below.

**GDH from T. thermophilus Shares a Similar Leucine-induced Allosteric Mechanism with Mammalian GDH**—It is well known that mammalian GDH is activated by leucine; however, neither the mechanism nor binding site has been elucidated. In both mammalian and T. thermophilus GDHs, leucine enhances the turnover number but does not decrease the $K_m$ value for substrates in the regulation. These observations suggest that both GDHs share a similar allosteric mechanism. To examine the generality of the leucine-mediated allosteric activation, we compared the amino acid sequences and three-dimensional structures of GDH50 enzymes of various organisms.

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**TABLE 1**

| Amino acid position in GdhA | 38 | 71 | 72 | 134 | 166 | 170 | 415 | 418 |
|----------------------------|----|----|----|-----|-----|-----|-----|-----|
| T. thermophilus GdhA Y     | I  | A  | R  | D   | M   | R   | Y   |
| T. thermophilus GdhB T     | A  | R  | M  | Y   |     |     |     |
| T. maritima GDH V           | V  | A  | R  | D   | M   | R   | Y   |
| B. subtilis RocG L           | D  | A  | G  | D   | R   |     |     |
| C. symbiosum GDH R           | G  | A  | A  | G   | K   | Q   | A   |
| C. glutamicum GDH R          | S  | A  | S  | G   | R   | Q   | A   |
| E. coli GDH S                 | S  | A  | A  | G   | K   |     |     |
| Bovine GDH I                | Q  | H  | R  | D   | S   | A   | F   |
| hGDH2 I                      | Q  | H  | R  | D   | S   | A   | F   |

(continued)
acid residues responsible for leucine binding in \textit{T. thermophilus} GDH are well conserved in \textit{T. maritima} GDH, suggesting a similar allosteric mechanism, possibly by leucine and/or the other hydrophobic amino acids in \textit{T. maritima} GDH (Table 1). In contrast, other bacterial GDHs, such as GDHs from \textit{Bacillus subtilis}, \textit{C. symbiosum}, \textit{Corynebacterium glutamicum}, and \textit{E. coli}, showed no apparent amino acid conservation at the corresponding positions; therefore, we assume that these GDHs do not bind amino acids in the corresponding regions.

Interestingly, Asp-166 and Arg-134, which directly recognize the \(\alpha\)-amino and \(\alpha\)-carboxyl groups of leucine in the GdhA-GdhB-Leu complex, are conserved in human and bovine GDHs (Table 1). Although the corresponding residues are located somewhat apart from each other in the apo-form (Fig. 5A), it is possible that these residues dislocate to bind leucine when leucine is added. In mammalian GDHs, although the residues at the positions necessary for recognition of the leucyl side chain are replaced with different types of residues, there is still space for binding leucine. The comparison of amino acid sequences and structures suggests that \textit{T. thermophilus} and mammalian GDHs share an allosteric activation mechanism by leucine. To examine this hypothesis, we expressed hGDH2, which is subject to prominent allosteric activation by leucine (29), and mutants at the leucine-binding site in \textit{E. coli} cells and analyzed their leucine response. The reductive amination activity for 2-oxoglutarate of hGDH2 was increased by 3.96-fold by the addition of 10 mM leucine (Fig. 6A). As expected from the structural comparison, replacement of Arg-151 corresponding to Arg-134 in GdhA of \textit{T. thermophilus}, which recognizes the \(\alpha\)-carboxyl group of leucine, with Met caused complete loss of sensitivity to leucine (Fig. 6B). Furthermore, replacement of Asp-185 corresponding to Asp-166, which recognizes the \(\alpha\)-amino group of leucine, with Ala made hGDH2 insensitive to leucine (Fig. 6C). These results suggest that these residues directly recognize leucine in hGDH2, sharing the regulatory mechanism with \textit{T. thermophilus} GDH. The R151M and D185A mutations affected the specific activity: 0.37 units/mg for the wild-type enzyme, 0.029 units/mg for R151M, and 0.26 units/mg for D185A. This observation suggests that a change in the environment in the subunit interface may affect the GDH activity, similar to the cases in the GDH mutants of \textit{T. thermophilus}.

Evidence supporting our hypothesis was recently reported. Inhibitors of bovine GDH were isolated by high-throughput screening (30), and crystal structures of complexes with three compounds, hexachlorophene, GW5074, and bithionol, were determined (31). In these structures, the inhibitors are bound in the interior space between the interfaces of the subunits, although the interaction sites are different between the complexes; hexachlorophene is bound to the inner core of the hexamer, whereas GW5074 and bithionol bind halfway between the core and the exterior of the hexamer (Fig. 5B). Interestingly, GW5074 and bithionol are bound at positions very close to leucine-binding sites in \textit{T. thermophilus} GDH; for example, the phenolic ring of bithionol stacks with Arg-147 in bovine GDH (Fig. 5C), which corresponds to Arg-134 in \textit{T. thermophilus} GDH. Accompanied by the stacking with Arg-147, binding bithionol causes displacement of Asp-181 and Arg-147 (corresponding to Asp-166 and Arg-134, respectively, of GdhA in the GdhA-GdhB-Leu complex) to positions similar to those of Arg-134 and Asp-166 in the GdhA-GdhB-Leu complex (Fig. 5C). Binding of these inhibitors inhibits bovine GDH by decreasing the \(V_{\text{max}}\) value (31), whereas binding of leucine increases the...
$V_{\text{max}}$ value of \textit{T. thermophilus} GDH (13). It is presumed that the internal core of GDH contracts when the catalytic cleft closes during the catalytic cycle; therefore, we assume that ligands, which can be bound to the subunit interfaces of GDH, can become activators or inhibitors by affecting domain opening and closing during the catalytic cycle allosterically. From this presumptive evidence, it is likely that mammalian GDH binds leucine at the subunit interfaces for allosteric activation and that leucine-mediated allosteroy might be common between mammalian and \textit{T. thermophilus} GDHs.

**Biological Implication**—By carrying heterohexameric GDH, which provides three different types of leucine-binding sites, \textit{T. thermophilus} GDH can make complicated responses to a wide range of amino acids in the environment. In particular, because GDH activity is affected by various hydrophobic amino acids, the physiological advantage of the regulation is still unclear at this time. One possible model is that an excess of effective amino acids, including leucine, activates GDH activity by binding hydrophobic amino acids, including leucine, at the subunit interfaces for allosteric activation and that ligands, which can be bound to the subunit interfaces of GDH, can become activators or inhibitors by affecting domain opening and closing during the catalytic cycle; therefore, we assume that ligands, internal core of GDH contracts when the catalytic cleft closes.

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