A New Class of Glutamate Dehydrogenases (GDH)

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF THE FIRST MEMBER, THE AMP-REQUIRING NAD-SPECIFIC GDH OF STREPTOMYCES CLAVULIGERUS

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Glutamate dehydrogenases (GDHs)1 are a broadly distributed group of enzymes (1, 2) that catalyze the reversible oxidation/dative deamination of glutamate to α-ketoglutarate and ammonia according to Reaction 1.

\[
glutamate + \text{NAD}(P)^+ + H_2O \rightleftharpoons \alpha\text{-KG} + \text{NAD}(P)H + H^+ + \text{NH}_4^+ \]

Reaction 1

Accordingly, GDHs, as well as aminotransferases, are enzymes that participate in the reassociation of α-amino groups inside cells, playing a central role in the regulation of the flux of intermediates between different biosynthetic and catabolic pathways (3, 4).

The GDHs identified in lower eukaryotes, or in prokaryotes, usually act with one particular coenzyme (NAD+ or NADP+), whereas those of higher eukaryotes have a dual coenzyme specificity (NAD(P)-GDH, EC 1.4.1.3) (5). In line with the generally observed biochemical rules for oxidative catabolic steps and reductive biosynthetic steps, NAD-GDHs (EC 1.4.1.2) are usually geared to catabolism of glutamate (6), and by contrast, NADP-GDHs (EC 1.4.1.4) are anabolic enzymes engaged in ammonia assimilation (7).

All GDHs reported to date are oligomeric enzymes (1–3) and, depending on the subunit size and monomer composition, can be grouped into the following two different structural types: hexameric GDHs (with six identical subunits of about 50 kDa) and tetrameric GDHs (with four identical subunits having a molecular mass close to 115 kDa). The hexameric type includes the NAD- or NADP-GDHs from most known bacterial species (8), the NADP-GDHs from lower eukaryotes (9), and the NAD(P)-GDHs from vertebrates (10). The tetrameric type includes the NAD-GDHs found in lower eukaryotes (9). Dehydrogenases that utilize glutamate, leucine, valine, and phenylalanine have been recognized as common members of a large superfamily (11–12). Thus, in line with proper hierarchical ordering, GDHs should be considered as a family of enzymes that includes two subfamilies as follows: small GDHs (S_GDHs, which contain the two member classes 50 kDa GDHs and 50 kDa GDHs) and large GDHs (L_GDHs, which until now contained only the 115 kDa GDHs). Some interesting considerations of the evolutionary relationship between tetrameric and hexameric GDHs have been ventured (13–14), and this topic area can now be reevaluated and expanded with the current discovery of a more diverse and complex GDH membership than previously known.

Despite the fact that GDH has an ubiquitous distribution in nature, this enzyme had not been previously detected in the important industrial species Streptomyces clavuligerus (15). For this reason, it was proposed that the catabolism of glutamic acid in S. clavuligerus does not require the participation of this
enzymatic activity (15). In contrast with the latter conclusion, we found that when the assimilation of glutamic acid by *S. clavuligerus* cultured in a medium containing this amino acid as the carbon and nitrogen source was studied, a GDH activity was, in fact, present. Here we report the purification, characterization, and sequence of the encoding gene of this enzyme, thus providing a first documentation of a previously unknown class of GDH. Phylogeographic and evolutionary relationships are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Molecular biology products were supplied by Amersham Pharmacia Biotech. Biochemicals and reagents were obtained from Sigma.

**Strains and Culture Media**—*S. clavuligerus* NRRL-3585 (ATCC 27084) was obtained from the American Type Culture Collection. Spores were produced as reported (16) and kept frozen at −20 °C in 20% w/v glycerol (17). DNA manipulations and sequence analysis were carried out as reported previously (18).

The medium used for the growth of *S. clavuligerus* was a seed medium (19) containing glycerol (10 g liter−1) and/or glutamic acid (5 g liter−1) as carbon sources. Incubations were carried out as reported (20).

**GDH Assay—** GDH activity (reductive amination) was assayed at 30 °C by following the oxidation of NADH at 340 nm in the presence of α-ketoglutaric acid and ammonia (21–23). The reaction mixture contained α-ketoglutarate (15 mM); NH₄Cl (25 mM); NADH (150 μM), the essential activator AMP (1 mM), and 25 μl of enzyme solution (between 4 and 15 milliunits). All substrates were dissolved in 100 mM HK₂PO₄/HNaPO₄ at pH 7.0. In some experiments, effectors were added to the assay mixture as specified. GDH reactions (1 ml) were started by adding α-ketoglutarate to the assay mixture, and the transformation of NADH into NAD⁺ was followed at 340 nm in a Shimadzu UV-120-02 spectrophotometer. One international unit (IU) of enzyme activity is defined as the catalytic activity leading to the consumption of a micro-mole of NADH per min. Usually, the activity is given as milliunits (10−3 IU) units. Specific activity is indicated as milliunits/mg protein.

When required, GDH activity was assayed in the direction of oxidative deamination. In such cases the concentrations of glutamic acid and NAD⁺ were 15 and 2 mM, respectively. Protein was measured by the method of Bradford (24).

**Purification of GDH from *S. clavuligerus*—** *S. clavuligerus* mycelia cultured as above (19–20) were harvested after 78 h of growth, filtered, and washed with sterile saline solution (1). Wet cells (25 g obtained from 3 liters of culture) were resuspended in 200 ml of 25 mM phosphate medium (19) containing glycerol (10 g liter−1) and assayed for GDH activity. The enzyme eluted between fractions 78 and 83, showing a peak in tube 81. By using this procedure we found that when the assimilation of glutamic acid by *S. clavuligerus* as the carbon and nitrogen source was studied, a GDH activity was, in fact, present. Here we report the purification, characterization, and sequence of the encoding gene of this enzyme, thus providing a first documentation of a previously unknown class of GDH. Phylogeographic and evolutionary relationships are discussed.

**RESULTS AND DISCUSSION**

**Physiological Aspects—** *S. clavuligerus* is a filamentous bacterium belonging to the Actinomycetales order, a group of microbes that is associated with broad biotechnological applications and of great industrial interest (35). Most of the strains included in the genus produce a variety of secondary metabolites that have clinical and pharmaceutical relevance (36). For example, *S. clavuligerus* synthesizes different β-lactam compounds, including penicillins, cephamycins, and the β-lactamase inhibitor clavulanic acid (37–38). The biosynthetic pathways of these compounds are strictly regulated, and most of them are under a mechanism of control directly related to the carbon or nitrogen source supplied to the medium (19, 39–41). Many efforts have been made to elucidate their biosynthetic pathways as well as the molecular basis of the regulatory mechanisms controlling them (19, 40–43). It has been shown that β-lactam antibiotic production is regulated by the nitrogen source used for growth in *S. clavuligerus* (34, 44). Accordingly, the influence of several enzymes related to nitrogen metabolism (GDH, glutamine synthetase, or glutamate synthase) on the biosynthesis of such compounds has been studied (15, 45–46). Although attempts to assay GDH in *S. clavuligerus* were unsuccessful (15), glutamine synthase and glutamate synthase have been studied and partially characterized (15, 45–46). In light of these results, it has been suggested that in *S. clavuligerus* the function of GDH is performed by alanine dehydrogenase, an enzyme that is also involved in nitrogen catabolism (15).

In contrast to the latter results, we observed the presence of a GDH that catalyzes in vitro the conversion of α-ketoglutarate and ammonia into glutamate (reductive amination) as well as the reverse reaction (oxidative deamination). The enzyme was...
found to be induced under nutritional conditions that demand the catabolic function (Fig. 1). This enzyme is NAD-specific, requires AMP as an essential activator, and it is strongly inhibited by Tris. These unusual characteristics undoubtedly explain why GDH was not detected before in S. clavuligerus. A chemically defined medium containing glycerol and glutamic acid as the carbon and nitrogen sources, respectively, supports excellent growth of S. clavuligerus (Fig. 2). Under these culture conditions, GDH achieved a maximal specific activity in the early phase of growth, and this level was sustained throughout the exponential phase of growth and even for a significant portion of the stationary phase. After an elapsed time of 100 h, rapid enzyme degradation was apparent. In view of these results, bacterial cultures were harvested at 78 h for the purification of GDH (see Table I).

Physicochemical and Enzymological Properties—Purified GDH (see Table I) migrated in SDS-10% PAGE as a single band with a molecular mass of 179 ± 7 kDa (Fig. 3). According to Sepharose CL-6B column chromatography, the native enzyme has a mass of 1,084 ± 55 kDa. PAGE analysis, carried out under non-denaturing conditions, revealed a molecular mass of 1,135 ± 39 kDa (data not shown). These results indicate that the native form of the enzyme is probably a homohexamer. By contrast, all the hexameric GDHs reported to date (NAD- or NADP-GDHs) have a monomeric molecular mass of about 50 kDa (14, 47). Therefore, GDH from S. clavuligerus represents a new class of glutamate dehydrogenase, i.e., a second class within the large subfamily of GDHs (L_GDHs, see below), designated as GDH L_180.

The catalytic activity of GDH was maximal at 30 °C (Fig. 4a). Similar optimal temperature values have been reported for the glutamate dehydrogenase of Bacillus cereus (48), Bacteroides fragilis (49), and Paracoccus denitrificans (50), whereas other homologs show higher optimal temperatures (51–52). S. clavuligerus GDH exhibited its maximal rate of catalysis at pH 7.0 (Fig. 4b), which is rather typical of GDHs (53), although examples of higher pH optima are also known (51).

Under the above conditions, the reactions were linear between 1.5 and 22 min when the quantities of GDH added to the assays ranged between 20 and 1 milliunits, respectively (data not shown). When substrate saturation profiles were obtained, GDH showed hyperbolic behavior toward increasing ammonia concentration (Fig. 5c) but sigmoidal responses to increasing changes in both α-ketoglutarate (Fig. 5a) and NADH (Fig. 5b) concentrations. Recently, the GDH of Psychrobacter sp. TAD1 was reported to exhibit similar phenomena of positive cooperativity (54). Unlike the S. clavuligerus GDH, the Psychrobacter GDH is NADP-specific and is a member of the S_50I class. It is perhaps of interest that in correlation with the positive cooperativity of substrate binding, the Psychrobacter GDH is thus far the only GDH to share the variant Rossmann motif (GXGXXS) for dinucleotide binding that is typical of the GDH L_180 class. Interestingly, ammonia and NADH caused inhibition of GDH above a given threshold concentration. Thus, the addition to standard reactions (in the absence of Asp, see “Experimental Procedures”) of NH₄⁺ at a concentration higher than 100 mM caused inhibition of GDH activity (about 10%), whereas if the concentration was 350 mM or higher, no activity was detected. Likewise, NADH began to inhibit GDH activity at a concentration of 200 μM (10%) whereas at 500 μM, inhibition exceeded 95%. Similar data were noted for GDH of Azospirillum brasilense (55). As occurs with other GDHs (51), the reductive amination reaction of S. clavuligerus GDH is inhibited by the reaction products (NAD⁺ and glutamic acid) as well as by NADP⁺ (see Table III).

Further kinetic studies allowed the calculation of the K_m, S_0.5 and Hill coefficient values in each case (see Table II). Additional analysis revealed that aspartic acid (see Fig. 5, a and b, and Table III) and asparagine (Table III) are allosteric activators of S. clavuligerus GDH. Although it has been reported that different molecules (AMP, ADP, GTP, ATP, NADP⁺, and succinate) could be allosteric modulators of different GDHs (51, 56–58), to our knowledge, this is the first description of aspartic acid and asparagine as allosteric activators of glutamate dehydrogenase.

In order to characterize the substrate specificity of this enzyme, some of the substrates of GDH were replaced during the assay by close structural molecules. Thus, when α-ketoglutarate (reductive amination) or glutamic acid (oxidative deamination) was replaced by other α, β, or γ ketoacids or by different amino acids (those indicated in Table III), respectively, no GDH activity was detected. This indicates specificity of the enzyme for α-ketoglutarate and for glutamic acid. Moreover, when NAD(H) was replaced by NADP(H), no catalysis was detected, therefore indicating that this enzyme is an NAD(H)-specific GDH.

In other experiments, AMP was omitted from the assays or replaced by other nucleotides. In its absence, no GDH activity was detected, indicating that AMP, at least in the assay conditions tested, is an essential activator for this enzyme. Although a similar activating effect of AMP on various GDHs has been observed elsewhere (53, 57, 59), AMP has not previously been found to be essential for activity. In Escherichia coli AMP is an activator at micromolar concentrations, but it is an inhib-
The purification was carried out as described in the text, starting from 25 g (wet weight) of cells.

| Purification step                  | Volume (ml) | Amount of protein (mg) | Enzyme activity (units) | Specific activity (units/mg protein) | Yield of recovery (%) | Purification fold |
|------------------------------------|-------------|------------------------|-------------------------|-------------------------------------|-----------------------|-------------------|
| Crude extract                      | 205         | 864                    | 81.4                    | 0.094                               | 100                   | 1                 |
| Ammonium sulfate precipitation     | 170         | 150                    | 66.4                    | 0.442                               | 81.6                  | 4.7               |
| (35–47.5%)                         |             |                        |                         |                                     |                       |                   |
| DEAE-Sephacel eluate (gradient     | 75          | 9.15                   | 30.0                    | 3.281                               | 36.9                  | 34.8              |
| 0.18–0.28 M KCl, ml 50–125)        |             |                        |                         |                                     |                       |                   |
| Sepharose CL-6B eluate (ml 474–498)| 24          | 0.42                   | 8.9                     | 21.195                              | 11.0                  | 225               |

Fig. 3. Electrophoretic mobilities in SDS-10% PAGE of purified GDH from *S. clavuligerus* (lane 2) and different proteins (lanes 1 and 3) used as *M*<sub>e</sub> standards (see “Experimental Procedures”).

Fig. 4. Effect of temperature (a) and pH (b) (●, 0.1 M citrate buffer; ■, 0.1 M phosphate buffer; ▲, 0.1 M glycine/NaOH buffer) on GDH activity.

| Enzymatic activity Specific activity Yield of recovery Purification |
|---------------------------------------------------------------|
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|---------------------------------------------------------------|
| Crude extract Crude extract                                  | 205         | 864                    | 81.4                    | 0.094                               | 100                   | 1                 |
| Ammonium sulfate precipitation Ammonium sulfate precipitation | 170         | 150                    | 66.4                    | 0.442                               | 81.6                  | 4.7               |
| (35–47.5%) DEAE-Sephacel eluate (gradient | 75          | 9.15                   | 30.0                    | 3.281                               | 36.9                  | 34.8              |
| 0.18–0.28 M KCl, ml 50–125) Sepharose CL-6B eluate (ml 474–498) | 24          | 0.42                   | 8.9                     | 21.195                              | 11.0                  | 225               |

Acid are also inhibitors (see Table III). Reports of inhibition caused by different central metabolic intermediates on GDH activities have appeared in the literature a number of times, but no common pattern of inhibition has been established (58, 60).

Enzymatic assays of GDHs are usually performed either in Tris-HCl or in phosphate buffer (22–23). However, we observed that the GDH activity of *S. clavuligerus* was not detected when cell-free extracts of this bacterium were prepared in 100 mM Tris-HCl. These data suggested that Tris could act as an inhibitor of the GDH. Analysis of this compound as an effector of GDH activity revealed that 50 mM Tris causes 81% of inhibition, whereas at 80 mM Tris, no activity was detected. Furthermore, other Tris structural analogs (or close related molecules) caused a similar degree of inhibition. Thus, when added as effectors to the GDH assay mixture (at a concentration of 50 mM), ethylamine, aminooxypropanol, ethanolamine, methoxyethylamine, and aminooxypropanediol caused inhibition of up to 60% (Table III). The fact that AMP is an essential activator together with the inhibition caused by Tris very likely explain previous failures to detect this enzymatic activity in *S. clavuligerus* cell-free extracts (15).

Isolation and Characterization of the Gene Encoding GDH in *S. clavuligerus*—The molecular mass of the GDH, deduced from the amino acid sequence encoded in the *gdh* is, 183,354 Da, in agreement with the molecular mass estimated for each monomer by SDS-PAGE (179 ± 7 kDa, see above).

The gene encoding GDH in *S. clavuligerus* (*gdh*) was isolated from a λ-GEM-12 genomic library using a very simple and efficient strategy (see “Experimental Procedures”) that allows the rapid amplification of a gene from a genomic library with the only requirement being that an oligonucleotide deduced from the amino terminus of a protein (for the whole gene) or deduced from an internal part of the protein (for the partial amplification of the gene) is known (28). By using this method, we PCR-amplified different bands that, when sequenced, revealed that they include an ORF of 4,953 base pairs (*gdh* gene) encoding a protein of 1,651 amino acid residues (Fig. 6). Analysis of the amino-terminal sequence (MQTKIDEAKaELARAARv) of the purified protein as well as other internal peptides isolated after tryptic digestion (AAVAADLVHEIALASSGR and VVEGEGGNLGLTQLGR) confirmed that all were present in the protein (see Fig. 6).

Furthermore, comparative analysis of the amino acid sequence of this protein with those included in the data bases without a functional assignment revealed that the central domain is quite similar to known GDHs, suggesting that the protein encoded by this gene is capable of GDH function, as expected. Additionally, a strong similarity (see Table IV) throughout the entire sequence was observed when *S. clavuligerus* GDH was compared with presumptive proteins encoded by genes present in the genomes of species belonging to the genera *Mycobacterium*, *Rickettsia*, *Pseudomonas*, *Vibrio*, *Clostridium difficile* is also inhibited by AMP, as well as by ADP and ADP-ribose (52).

When AMP was replaced by ADP, 20% of activity was detected, but when AMP was substituted by other mono-, di-, or trinucleotides, no activity was measured. Furthermore, when ATP and GTP were tested as effectors, they inhibited GDH activity (Table III). Vertebrates possess a GDH that is subject to allosteric inhibition by GTP (57). Study of the influence of other molecules on the GDH activity revealed that some intermediates of the Krebs cycle (isocitrate, succinate, fumarate, malate, and oxalacetate) as well as glyoxylic acid and glutaric acid are also inhibitors (see Table III). Reports of inhibition caused by different central metabolic intermediates on GDH activities have appeared in the literature a number of times, but no common pattern of inhibition has been established (58, 60).
Shewanella, and Caulobacter. Although the genes encoding all these proteins showed low percentages of identity, the ORFs from Rickettsia prowazekii, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas putida, Pseudomonas aeruginosa, Vibrio cholerae, Shewanella putrefaciens, and Caulobacter crescentus encode putative GDHs showing a similar size (~180 kDa) and a high percentage of identity in their amino acid sequences (see Table IV).

It seems readily apparent that the GDH of S. clavuligerus represents a new class of GDH exhibiting some breadth of distribution in nature. This hexameric GDH has a monomer size that corresponds to neither the typical 50-kDa size of all the hexameric GDHs reported to date nor to the 115-kDa size of tetrameric GDHs. Thus, according to the criteria for oligomeric structure and monomer size, three different types of GDHs (α6-50, α6-180, and α4-115 kDa) exist. Hierarchical homology grouping (see below) reveals the existence of four different well defined classes (50I, 50II, 115, and 180 kDa). These sort into two widely spaced subfamilies as follows: the small GDHs (S_GDHs, including 50I and 50II classes) and the large GDHs (L_GDHs, including 115 and 180 GDHs classes). The structural
The relationship between the hexameric SGDH subfamily and the tetrameric GDH L115 class has been assessed (14). Insertions and deletions that comprise the essential alignment variations were found to be between or at the ends of the elements of secondary structure. Substantial insight has been obtained into the understanding of the relationship between secondary structure, the variation in particular sequence, and the unique insertions/deletions on the one hand, and with the subunit interactions that dictate oligomer size on the other hand. The eventual crystallographic structure of the GDH L180 protein will be a fascinating addition to this analysis because it is hexameric (similar to the less closely related S_GDH subfamily members), yet it is much more similar to the tetrameric GDH L115 class members in terms of overall identity and the gen-

**Fig. 6.** Nucleotide sequence of gdh, amino acid sequence and key GDH residues of deduced GDH of *S. clavuligerus*. Underlined sequences correspond to oligopeptides obtained by tryptic digestion of the native protein. Asterisks indicate a putative Shine-Dalgarno box. The central portion of this protein is the GDH domain, and it is flanked by domains of unknown function. The GDH domain itself has two subdomains as follows: the glutamate-binding region (subdomain I) and the dinucleotide-binding region (subdomain II). Subdomain I is indicated as a region with gray color; subdomain II is indicated with yellow color; glutamate-binding residues are in red; residues contacting the dinucleotide are indicated in blue, and residues contacting both glutamate and dinucleotide (K in subdomain I and N and G in subdomain II) are indicated in black boxes.
**Glutamate Dehydrogenase of S. clavuligerus**

**Table IV**

| Organisms       | No. | Amino acids | Mass | Identity | Accession data bank |
|-----------------|-----|-------------|------|----------|---------------------|
| *S. clavuligerus* | 1   | 1,651       | 183,354 | 100 | AF218569            |
| *C. crescentus*  | 2   | 1,607       | 176,496 | 40.8 | TIGR                |
| *P. aeruginosa*  | 3   | 1,620       | 182,637 | 43.5 | EMBL                |
| *P. putida*      | 4   | 1,621       | 182,491 | 43.2 | TIGR                |
| *S. putrefaciens*| 5   | 1,614       | 182,332 | 45.0 | TIGR                |
| *V. cholerae*    | 6   | 1,613       | 183,359 | 42.7 | TIGR                |
| *M. tuberculosis*| 7   | 1,624       | 176,899 | 43.5 | CAA16053            |
| *M. bovis*       | 8   | 1,623       | 176,798 | 43.5 | TIGR                |
| *M. avium*       | 9   | 1,617       | 177,097 | 41.8 | TIGR                |
| *M. leprae*      | 10  | 1,622       | 177,875 | 43.5 | CAB43156            |
| *R. prowazekii*  | 11  | 1,581       | 181,383 | 28.7 | CAA15186            |

**Table V**

Comparison of critical C-5 carbon contacts of glutamate within different GDH classes

The 1st column includes both classes of GDH S_50, and residue numbers are those of *C. symbiosum* as the reference organism. The equivalent residue numbers are given in the 3rd column for *S. clavuligerus* in parentheses. Arrows indicate parallel conserved changes in the two GDH_L classes in comparison with the residues indicated in the 1st column.

| 50-kDa GDHs | 115-kDa GDHs | 180-kDa GDHs |
|-------------|--------------|--------------|
| C-5 carboxyl interacts with NH$_3^+$ | Lys-89 | Arg-98 | Arg-98 (Arg-849) |
| C-5 carboxyl interacts with β-OH | Thr-193 | Thr-193 | Ser-380 (Ser-78) |
| C-5 carboxyl interacts with β-OH | Ser-380 (Ser-78) | Gly-163 (Gly-376) |
| C-5 carbon interacts with side chain | Ala-163 (Ala-376) | Val-377 (Val-777) |

A classical βαβ fold for dinucleotide binding is shown near the amino-terminal portion of subdomain II. The typical GXXGXXG motif is replaced by GXXGXS in *S. clavuligerus*. Overall in the L_180 group this motif is either GXXGXXS or GXXGXXA. Ahead of the aforementioned Rossmann fold is a conserved threonine 992 (Thr-209) that influences the conformation of the glycosidic bond of the nicotinamide ring. The latter together with Gly-1206 (Gly-376), Val-1207 (Val-377), and Ser-1210 (Ser-380) dictate the stereospecificity of the hydride transfer. In this region Asn-1203 (Asn-373) hydrogen bonds to Lys-875 (Lys-113) across the GDH domain interface.

We note that analysis of the active site residues in the Glu/Leu/Val dehydrogenase superfamily using the program PROSITE has resulted in identification of the consensus sequence (LIW)XXGG(SAG)XX(XV)XXX(DNST)PL (PS00074). The lysine residue corresponds to *S. clavuligerus*...
residue 885 marked in Fig. 7. The GDH L_{180} class does not fit the pattern perfectly, the corresponding residues being \( \text{(I)} \text{XX} ^{\text{vG(AS)K}} \text{X} ^{(G)} \text{XXX} ^{(kNr)(rfqk)} \). The lowercase residues represent ones that do not fit the consensus. An inspection of Fig. 7 shows that the region of greatest absolute conservation of neighboring residues is in a region of primary sequence where both glutamate and dinucleotide contacts are made. In this region (around \text{*S. clavuligerus* residue 1210 marked in Fig. 7) the consensus pattern is \( \text{N} \text{XX} ^{\text{GV}} \text{XX} ^{\text{S}} \text{XX} ^{\text{E}} \).

**Beyond the Core GDH**—The core GDH is quite well defined (61–66) with respect to the atomic interactions that dictate glutamate binding (within subdomain I) and dinucleotide binding (within subdomain II). Detailed structural information about interface regions that are responsible for oligomerization within subdomain I are also elucidated. Beyond the structural basis for catalytic competency and for oligomerization, some insight is emerging about the structural basis for regulatory properties. Different GDH types can be quite variable with
respect to whether they are sensitive to allosteric control. Where such control exists, marked variety is evident with respect to the complexity of the control. The mammalian GDHs exemplify a case where complex effector control is accompanied by a somewhat larger size (about 10%) of the monomer (66). An insertion of about 40 amino acids is placed just beyond the carboxyl-terminal end of the core GDH region, the end point of the alignment shown in Fig. 7 (i.e., the novel insert begins at about residue 454 for the *Homo sapiens* GDH). This 48-amino acid insert has been described as an antenna that serves as an intersubunit communication conduit and intimately influences negative cooperativity and allosteric regulation promoted by GTP, NADH, and ADP.

*Chlorella sorokiniana* exemplifies another case where additional sequence is associated with regulatory complexity (33). In this most fascinating of systems, an amino-terminal extension consisting of two additional α-helices (relative to *C. symbiosum*) exist. Differential transcript splicing yields two subunit types, differing only at the amino terminus. The two homohexamer types differ dramatically in the affinity for ammonia in the reductive amination activity. Differential regulation of the relative levels of the two subunit types, derived from a single gene, provides the potential for the fine-tuned modulation of α-ketoglutarate amination to an impressive degree since all ratios of heterohexamers presumed to have intermediate catalytic properties can be generated. The function of the large amount of amino acid sequence that flanks the GDH domain of both GDH L-115 kDa and now GDH L-180 is completely unknown. Aside from the similarity with other members of the same GDH class, these regions showed no similarity
with any other proteins in the data base. No recognizable motifs or membrane-spanning regions were detected. Since the GDH L180 of S. clavuligerus exhibits an extensive complexity of regulatory properties, it seems quite possible that some or all of the regions flanking the GDH domain may exist for regulation.  

Relationships between the Four Homolog Classes of GDH—To establish the phylogenetic relations between the different GDHs reported to date, a comparative study of all these proteins (Tables IV and VI) was performed. Fig. 8 illustrates an unrooted tree obtained by the neighbor-joining methodology of all complete sequences available (see Fig. 7). At difference with the analysis reported in the present study, four different GDH groups were established, reinforcing the convenience of defining two subfamilies between GDHs, since the GDH motifs in S50I and S50II differ from each other just about as much as L115 and L180 (see Fig. 7).

Evolutionary History of GDH—Within subfamily L_GDHs, members of GDH L115 are found only in fungi and protozoans, whereas the phylogenetic range of GDH L180 is thus far restricted to bacteria. Within subfamily S_GDHs, members of GDH S50I are present in the bacteria, fungi, protozoans, and algae. Although higher plant chloroplasts apparently lack GDH, Chlorella possesses a thoroughly characterized chloroplast-localized species of GDH S50I (67). Members of the GDH S50II class are present in Archaea, Bacteria, mitochonidria of vertebrates, and mitochonidria of higher plants.  

Fig. 10 illustrates the phylogenetic distribution of GDH-encoding genes in selected members of the Archaea and Bacteria that have been organized on a 16 S rRNA tree. Since the genomes of most of these organisms are completely sequenced, one can be relatively confident not only that representatives of every known homolog class has been detected but that GDH genes not found really are absent. C. sorokiniana represents the position of chloroplast 16 S rRNA. Mitochondrial 16 S rRNA (not shown) would be appropriately at the divergence level of R. prowazekii. Class GDH S50II clearly exhibits the broadest level of phylogenetic distribution, being the only GDH representative present in Archaea. It follows that this is likely to have been the ancestral GDH species. The S50II species of GDH appeared early after divergence of the Bacteria and Archaea at about the level of Deinococcus (which has both paralogs). The joint presence of both paralogs is seen elsewhere, e.g. species of Neisseria and Bordetella. Although E. coli possesses the position of chloroplast 16 S rRNA. Mitochondrial 16 S rRNA (not shown) would be appropriately at the divergence level of R. prowazekii. Class GDH S50II clearly exhibits the broadest level of phylogenetic distribution, being the only GDH representative present in Archaea. It follows that this is likely to have been the ancestral GDH species. The S50II species of GDH appeared early after divergence of the Bacteria and Archaea at about the level of Deinococcus (which has both paralogs). The joint presence of both paralogs is seen elsewhere, e.g. species of Neisseria and Bordetella. Although E. coli possesses

with any other proteins in the data base. No recognizable motifs or membrane-spanning regions were detected. Since the GDH L180 of S. clavuligerus exhibits an extensive complexity of regulatory properties, it seems quite possible that some or all of the regions flanking the GDH domain may exist for regulation.  

Relationships between the Four Homolog Classes of GDH—To establish the phylogenetic relations between the different GDHs reported to date, a comparative study of all these proteins (Tables IV and VI) was performed. Fig. 8 illustrates an unrooted tree obtained by the neighbor-joining methodology of all complete sequences available (see Fig. 7). At difference with the analysis reported in the present study, four different GDH groups were established, reinforcing the convenience of defining two subfamilies between GDHs, since the GDH motifs in S50I and S50II differ from each other just about as much as L115 and L180 (see Fig. 7).

Evolutionary History of GDH—Within subfamily L_GDHs, members of GDH L115 are found only in fungi and protozoans, whereas the phylogenetic range of GDH L180 is thus far restricted to bacteria. Within subfamily S_GDHs, members of GDH S50I are present in the bacteria, fungi, protozoans, and algae. Although higher plant chloroplasts apparently lack GDH, Chlorella possesses a thoroughly characterized chloroplast-localized species of GDH S50I (67). Members of the GDH S50II class are present in Archaea, Bacteria, mitochonidria of vertebrates, and mitochonidria of higher plants.  

Fig. 10 illustrates the phylogenetic distribution of GDH-encoding genes in selected members of the Archaea and Bacteria that have been organized on a 16 S rRNA tree. Since the genomes of most of these organisms are completely sequenced, one can be relatively confident not only that representatives of every known homolog class has been detected but that GDH genes not found really are absent. C. sorokiniana represents the position of chloroplast 16 S rRNA. Mitochondrial 16 S rRNA (not shown) would be appropriately at the divergence level of R. prowazekii. Class GDH S50II clearly exhibits the broadest level of phylogenetic distribution, being the only GDH representative present in Archaea. It follows that this is likely to have been the ancestral GDH species. The S50II species of GDH appeared early after divergence of the Bacteria and Archaea at about the level of Deinococcus (which has both paralogs). The joint presence of both paralogs is seen elsewhere, e.g. species of Neisseria and Bordetella. Although E. coli possesses
only GDH S_50I (as does Yersinia pestis), other enteric bacteria (e.g. Klebsiella and Salmonella) possess both paralogs of GDH. The gene encoding GDH L_180 also appeared early after the divergence of Archaea and Bacteria, being present in both Gram-positive and Gram-negative bacteria. Streptomyces coelicolor possesses both GDH L_180 and GDH S_50II, whereas both P. aeruginosa and P. putida possess GDH L_180 and GDH S_50I. Thus far, no organism is known to have all three types of GDHs. Since S. coelicolor possesses GDH S_50II, it is possible that S. clavuligerus may also possess this so far undetected class. Whereas the emergence of the GDH S_50I paralog can be accounted for by a simple gene duplication and divergence scenario, the emergence of GDH L_180 requires a more complex explanation to account for the considerable increase in gene size.

It is not surprising that those organisms that have a minimal genome size that is associated with restricted metabolic repertoires (e.g. Mycoplasma, Ureaplasma, Chlamydia, Treponema, and Borrelia) lack all types of GDHs. In line with contemporary conclusions about genome reduction in such organisms, it seems certain that these organisms lost GDH genes in relatively recent times. Fig. 10 also shows the distribution of leucine/valine/phenylalanine dehydrogenases. These appear to be of relatively infrequent occurrence in the Bacteria. It is apparent from inspection of Fig. 8 that in the Bacteria there has been a dynamic progression of evolutionary events involving paralog acquisition and paralog loss. Bacillus subtilis possesses two copies of GDH S_50I. Even closely spaced phylogenetic progressions such as the gamma group of Proteobacteria (from P. aeruginosa to Salmonella typhi in Fig. 8) exhibit a

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**FIG. 8. Unrooted tree for GDH family.** The core GDH regions of a balanced representation of the four classes of GDH were subjected to maximum parsimony analysis. Bootstrap values obtained for 500 iterations are indicated at the branch nodes. The motif surrounding the active site lysine (Lys-875 in S. clavuligerus; Lys-113 in C. symbiosum) is indicated for each of the four GDH groups. The corresponding motif for the leucine/valine/phenylalanine family of DH is also shown at the lower left.
marked flux in the combination of GDH classes present.

An examination of the multiple alignment shown in Fig. 8 shows that GDH L115 and GDH L180 are sister classes within subfamily L_GDH, whereas GDH S50I and GDH S50II are sister classes within subfamily S_GDH. These relationships are specifically tied to the GDH domain. The non-GDH sequence in the GDH L180 class has no significant similarity with the non-GDH sequence in the GDH L115 class. The functions of the non-GDH regions in both classes of L_GDH are completely mysterious at present, but they must have evolved independently from a common ancestor having the unique sequence elements of the L_GDH subfamily. Within both of the GDH L180 and GDH L115 classes, the GDH domain is distinctly more conserved than the non-GDH regions. It is interesting that GDH L180 has retained the structural features that dictate the hexameric subunit configuration usually seen in the S_GDH subfamily, even though it has a much greater overall similarity with the GDH L115 class of GDH that exhibits a tetrameric subunit configuration.

Proposed Evolutionary Scenario—The most ancient GDH species is likely to have been GDH S50I. It is broadly distributed throughout the Archaea and Bacteria. It also is present in Eukaryotes where higher plants and vertebrates express it as a mitochondrion-localized species. This presumably was acquired via endosymbiosis, probably from a member donor within the α division of Proteobacteria. Following the divergence of Archaea and Bacteria, gene duplication produced GDH S50I at a time between the divergence of Thermotoga and Deinococcus. Even though the GDH species present in contemporary Chlorella chloroplasts and in contemporary Synechocystis are different classes of GDHs, the tree dynamics are clearly consistent with the presence of the Chlorella-type GDH in an ancestral cyanobacterium that may have had both GDH S50 classes. Indeed, cyanobacteria as a group are sufficiently diverse that one could reasonably expect to find both GDH S50 classes in other modern genera of cyanobacteria whose genomes are not yet sequenced. The phylogenetic divergence of different cyanobacteria exceeds the phylogenetic breadth of the enteric bacteria, where a considerable diversity of GDH distribution is apparent (Fig. 10).

An active-site motif of the GDH domain (see S. clavuligerus residue marked 875 in Fig. 7 for reference) is LXXXQXXKN in the GDH S50I class (except for Agaricus bisporus and C. symbiosum, which exhibit LXXXQXXKD). This motif varies in the GDH domain of class GDH S50II where it is LXXXMXXXKc/t (except for S. sulfataricus which is LXXXMXXXKN). We suggest that this motif is a molecular flag (as depicted by the distribution of motif variation in Fig. 8) that reflects the evolutionary steps leading to the contemporary GDH phylogenetic distribution (Fig. 10). The L_GDH subfam-
ily uniformly exhibits the LXXXQXXKN motif present in the GDH S_50I class, and thus, we propose that GDH S_50I was the immediate precursor to the L_GDH subfamily. Subsequent fusions led to the increased sizes of the modern L_GDH subfamily; these fusions must have been of independent origin since no similarities of the non-GDH regions are apparent. GDH L_115 may have been acquired by lower eukaryotes and protozoans via endosymbiosis since nuclear genes with this origin do not necessarily make gene products targeted to intracellular organelles (68). So far, no member of the Bacteria has been found to possess a gene corresponding to the likely evolutionary intermediate, i.e. encoding an approximately 50-kDa GDH that corresponds more closely to the GDH domain of the L_GDH subfamily rather than of the S_GDH subfamily. The ancestral GDH S_50II species may have possessed broad substrate specificity. Indeed, broad substrate specificity is a property of some contemporary species of GDH S_50II (e.g. bovine GDH) (see Ref. 69 and references therein). The leucine/vale phenylalanine dehydrogenase families may have originated from GDH S_50II in the Gram-positive lineage via gene duplication followed by substrate specialization as formulated by the recruitment hypothesis (70). Consistent with this is the
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presence of LXXXMMXTK as an active-site motif for all leucine/valine/phenylalanine dehydrogenases (Fig. 7 and 8).

To summarize, this analysis reveals the following: (i) the nitrogen metabolism of S. clavuligerus involves a new type of GDH (genetically and biochemically characterized) that requires a new classification for the different types of GDHs; (ii) all GDHs reported up to date belong to two different subfamilies (small GDHs and large GDHs); (iii) each subfamily subdivides into two sister classes (S_50I or S_50 II and L_115 or L_180); and (iv) α_115 and α_180 may have had a closer evolutionary variation with the 50_1 (α_1) class (enzymes 18–46) than with the 50_2 (α_2) class (enzymes 47–77) (see Table VI).

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