Mutation of His<sup>465</sup> Alters the pH-dependent Spectroscopic Properties of <i>Escherichia coli</i> Glutamate Decarboxylase and Broadens the Range of Its Activity toward More Alkaline pH<sup>*</sup>

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Glutamate decarboxylase (GadB) from <i>Escherichia coli</i> is a hexameric, pyridoxal 5'-phosphate-dependent enzyme catalyzing CO<sub>2</sub> release from the α-carboxyl group of L-glutamate to yield γ-aminobutyrate. GadB exhibits an acidic pH optimum and undergoes a spectroscopically detectable and strongly cooperative pH-dependent conformational change involving at least six protons. Crystalllographic studies showed that at mildly alkaline pH GadB is inactive because all active sites are locked by the C termini and that the 340 nm absorbance is an aldamine formed by the pyridoxal 5'-phosphate-Lys<sup>276</sup> Schiff base with the distal nitrogen of His<sup>465</sup>, the penultimate residue in the GadB sequence. Herein we show that His<sup>465</sup> has a massive influence on the equilibrium between active and inactive forms, the former being favored when this residue is absent. His<sup>465</sup> contributes with n ≈ 2.5 to the overall cooperativity of the system. The residual cooperativity (n ≈ 3) is associated with the conformational changes still occurring at the N-terminal ends regardless of the mutation. His<sup>465</sup>, dispensable for the cooperativity that affects enzyme activity, is essential to include the conformational change of the N termini into the cooperativity of the whole system. In the absence of His<sup>465</sup>, a 330-nm absorbing species appears, with fluorescence emission spectra more complex than model compounds and consisting of two maxima at 390 and 510 nm. Because His<sup>465</sup> mutants are active at pH well above 5.7, they appear to be suitable for biotechnological applications.

Glutamate decarboxylase (Gad; EC 4.1.1.15) is a pyridoxal 5'-phosphate (PLP)-dependent<sup>2</sup> enzyme widely distributed among living organisms (1). It catalyzes CO<sub>2</sub> release from the α-carboxyl group of L-glutamate to yield 4-aminobutyrate (γ-aminobutyrate (GABA)). In commensal and pathogenic strains of <i>Escherichia coli</i> and in other enteric bacteria, like <i>Shigella flexneri</i>, <i>Listeria monocytogenes</i>, and <i>Lactococcus lactis</i>, Gad is a key component of the most important acid resistance system, based on glutamate. This system protects enteric bacteria from the extreme acid stress they encounter during transit through the stomach of the host on their way to the gut (2). The system exploits the proton consuming activity of Gad by replacing the leaving CO<sub>2</sub> with a proton, which is reversibly incorporated into GABA.

<i>E. coli</i> possesses two Gad isoforms, GadA and GadB, which exhibit an acidic pH optimum (pH 3.8–4.6) and become activated intracellularly because in extremely acidic environments protons leak through the bacterial cell membrane (2). pH-dependent activation of Gad is accompanied by a distinct change in the absorption spectrum of the cofactor (3–6). At wavelengths above 300 nm, the spectrum changes from one at pH > 5.3 with the major absorbance band at 340 nm (inactive enzyme) to another at pH < 5.3 with the major band at 420 nm (active enzyme). The midpoint of the spectroscopic change is shifted from pH 5.3 to 5.8 in the presence of chloride, which is the most abundant anion in gastric secretions (3). The spectral transition exhibits a high level of cooperativity of the protonation-deprotonation process, with at least six protons being involved (3–5). The 420-nm absorbing species is generally accepted to be the ketoenamine form of the PLP-Lys<sup>276</sup> internal aldimine, protonated on the Schiff base nitrogen (Fig. 1A). Two structures have been proposed for the 340-nm absorbing species (Fig. 1A). O’Leary and Brummund (7, 8) assumed that it is an aldimine in which the C4’ is substituted by a cysteine residue. Tramonti et al. (5), upon mutation of the Lys<sup>276</sup>, proposed that the 340-nm chromophore is the enolimine tautomer of the ketoenamine. Model studies in aqueous and nonpolar solvents have shown that aldmines and aldamines of PLP originate different fluorescence emission spectra when excited at 330 nm (9). The enolimine tautomer of the Schiff base with hexylamine emits maximally at 512–518 nm. Aldmines formed with histidine and cysteine show a single emission peak with a λ<sub>max</sub> value of 367–385 nm. This occurs because the C4’ of the cofactor is sp<sup>3</sup>-hybridized, and the conjugation between the double bond of the Schiff base and the pyridinium ring is lost (10). These properties were used to assign enolimine or aldamine structures in several PLP-dependent enzymes (9–15).

Several structures of GadB are available: high and low pH forms, halide-bound forms, and a mutant (GadBΔ1–14) lacking...
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![Chemical structures](image_url)

EXPERIMENTAL PROCEDURES

**Materials—FastStart High Fidelity DNA polymerase, restriction enzymes, alkaline phosphatase, and ampicillin were from Roche Applied Science, TOPO<sup>®</sup> TA cloning system was from Invitrogen, and the DNA ligation system and DEAE-Sepharose FF were from GE Healthcare. Taq DNA polymerase and SDS-PAGE protein markers were from Fermentas, and the plasmid DNA and the DNA extraction kit from agarose gel were from Nucleospin. Ingredients for bacterial growth were from Riedel-de Haen. 4-(2-Hydroxyethyl)piperazine-1-(3-propane-sulfonic acid) was from Acros Organics. Vitamin B6, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 1-γ-glutamic acid, and kanamycin were from Fluka. All other chemicals were from Sigma. Oligonucleotide synthesis and DNA sequencing services were by MWG Biotech.**

**Site-directed Mutagenesis—Construction of the E. coli GadB mutants was performed by PCR amplification on the entire gadB open reading frame as cloned in pQ<sub> gadB </sub>(16). In each case two primers were used. Site-directed mutagenesis for GadBH465A was performed using the forward oligonucleotide 5′-GGCGTATACGAGGCCCTTTC-3′, which anneals upstream of the pQE60 polycloning site, and the mutagenic oligonucleotide 5′-GGAAGCTTAAACGTATACGAGGCCCTTTC-3′. The deletion mutant GadBΔHHT, lacking His<sup>465</sup> and Thr<sup>466</sup>, was generated using the forward primer 5′-GGCCA7GGAATAAGAAGCTAGTAACG-3′, which anneals in the 5′ coding region of the gadB gene, and the mutagenic oligonucleotide 5′-GGAAGCTTAAACGTATACGAGGCCCTTTC-3′. The italicized sequences indicate the Ncol and HindIII restriction sites used for directional cloning of the PCR products into pQE60. The underlined nucleotides are the codon substitutions introducing the His → Ala mutation in GadBH465A, whereas the nucleotides in bold are those in between the two triplets coding for His<sup>465</sup> and Thr<sup>466</sup> that were deleted in GadBΔHHT. The amplification products were cloned in the pCRII-TOPO vector (TOPO<sup>®</sup> TA cloning system). E. coli Mach1-T1 transformants were selected by blue/white screening. Plasmids from white colonies were purified and fully sequenced on both strands. Plasmids pCRII-H465A and pCRII-ΔHHT were digested with EcoRV and HindIII, which cut 624 nucleotides upstream and 12 nucleotides downstream from the GadB TGA stop codon, respectively. The 639-nucleotide DNA fragment was subcloned into pQ<sub> gadB </sub>(16) digested with the same restriction enzymes and therefore lacking the C-terminal region of gadB. The ligation mixture was used to transform E. coli JM109/pREP4. Transformants were screened by colony PCR. Plasmids from positive clones were also digested with Ncol and HindIII restriction enzymes to confirm the presence of the entire mutated gene.**

**Protein Purification, SDS-PAGE, and Cell Fractionation—**The conditions used for expression and purification of GadBH465A and GadBΔHHT were essentially as described for wild type GadB (16) except that the DEAE-Sepharose chromatography was performed at 4 °C instead of at room temperature to improve the stability of the mutant enzymes.
Protein purity was judged by 12% SDS-PAGE (17). Enzyme concentration and activity were assayed as described (16). The PLP content of all preparations was determined by treating the proteins with 0.1 M NaOH and measuring absorbance at 388 nm ($\epsilon_{388} = 6550$ liter-mol$^{-1}$-cm$^{-1}$) (18).

The effect of pH on the cellular localization of GadBH465A and GadB∆HT compared with wild type GadB was established following cell fractionation. Cytoplasmic and membrane fractions from E. coli strains JM109/pREP4 containing either pQgadBH465A or pQgadB∆HT were obtained and assayed for enzyme activity and by immunoblot as described (4).

**Gad Activity Assay**—Two assay methods were used. The Gabase assay quantifies GABA production (16), and the specific enzyme activity and by immunoblot as described (4).

**RESULTS**

**Overproduction and Purification of GadBH465A and GadB∆HT**—His$^{465}$ of GadB was either replaced with alanine (GadBH465A) or deleted together with the last residue in the polypeptide chain, Thr$^{466}$ (GadB∆HT). Both mutants were overexpressed in E. coli and purified essentially following the protocol used for wild type GadB (16), which was also purified for comparison purposes. During DEAE-Sepharose chromatography at pH 6.5 the mutants, unlike wild type GadB, eluted as yellow bands, indicating that the mutations had altered their absorption spectra. The purity of both mutants was >95%, as based on SDS-PAGE (data not shown). The mutants were stable at 4 °C for many months. The yield from a standard purification (a 2-liter culture) was approximately 70 mg for GadBH465A and 43 mg for GadB∆HT, corresponding to 78 and 52% of the specific activity of the mutants, unlike wild type GadB (Fig. 2), but the catalytic efficiency ($k_{cat}$ and $K_m$) were determined in 50 mM sodium acetate buffer, pH 4.6, by following the changes in absorbance at 420 and 340 nm observed during the reaction of wild type GadB and of GadBH465A and GadB∆HT in the presence of different concentrations of L-glutamate as described (21). The absorption spectra were resolved into their component absorption bands (deconvolution) by nonlinear least square fit of the experimental data to the sum of a variable number of log normal curves, each having independent parameters (22).

**Bioinformatic Analysis**—A $K_a/K_s$ ratio analysis was carried out as follows: 14 orthologues/paralogues of GadB were manually selected from the output of a BLAST search versus the UniProt data base. The protein sequences were aligned with T-COFFEE (23), and the corresponding coding sequences were retrieved from the EMBL/CDSS data base (24) using DBFETC and aligned with REVTRANS (25) using the protein sequence alignment as template. $K_a/K_s$ ratios were calculated with SELECTON (26) in high accuracy mode using the M8 codon substitution model.
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FIGURE 2. UV-visible absorption spectra. Absorption spectra of the purified proteins were obtained in 50 mM sodium acetate buffer, pH 4.86. The spectra of wild type GadB (continuous line), GadBH465A (dashed line), and GadB\textsuperscript{HT} (dotted line) were normalized to a protein concentration of 7.7 \(\mu\)M. In the inset, the 300–500 nm region of the spectra is expanded.

### TABLE 1

| Protein         | \(k_{cat}\)  | \(K_m\)    | \(k_{cat}/K_m\) |
|-----------------|--------------|------------|-----------------|
| Wild type GadB  | 24.85 ± 0.13 | 2.32 ± 0.05| 10.71 ± 0.02    |
| GadBH465A       | 20.75 ± 0.06 | 1.51 ± 0.02| 13.74 ± 0.01    |
| GadB\textsuperscript{HT} | 16.24 ± 0.06 | 1.61 ± 0.03| 10.08 ± 0.02    |

The values reported were calculated using the integrated Michaelis-Menten equation to fit the curves of the disappearance of the enzyme-substrate complex as a function of time as described by Tramonti et al. (21).

of GadBH465A and GadB\textsuperscript{HT} changed very little in the range of pH 4.5–7.0 (data not shown). This behavior contrasts with that of wild type GadB (4, 5). The range of analysis was therefore extended to 9.5, and phosphate was used as buffer instead of acetate. The spectral changes shown by the mutants are different in several respects. In the wild type enzyme at pH 6.8, less than 10% of the 420-nm absorbance observed at pH 4.6 is present in several respects. In the wild type enzyme at pH 6.8, less than 10% of the 420-nm absorbance observed at pH 4.6 is still detected above the 300–500 nm region of the spectra is expanded. The absorption spectra at pH 4.6 and 6.8 (wild type) or 8.5 (GadBH465A and GadB\textsuperscript{HT}) were resolved into their component absorption bands (Fig. 4 and data not shown). Good fits were obtained to two components in all cases except for wild type GadB at pH 6.8 (Fig. 4A) where three components were required, with \(\lambda_{max}\) values of 330, 341.5, and 410 nm. Because the species absorbing at 341.5 nm predominates at pH 6.8 in wild type GadB and is absent from the spectra of the mutants, it is deduced to be the aldimine with His\textsuperscript{465}.

**CD and Fluorescence Properties**—The effects of mutating His\textsuperscript{465} on the CD and fluorescence spectra of GadB were investigated. The CD spectrum of GadBH465A at pH 4.6 displays a peak at 420 nm, coinciding in intensity and shape with that of wild type GadB (Fig. 5). At alkaline pH the CD spectra are significantly different; in wild type GadB the CD signal at pH 7.5 is essentially flat in the range 300–500 nm, whereas in GadBH465A at pH 8.3 the 420-nm signal is still present and accounts for more than 45% of the signal detected at pH 4.6 (Fig. 5). In addition GadBH465A at a pH level of >7.5 shows a positive CD signal centered at 332 nm. The CD spectra of GadB\textsuperscript{HT} were very similar to those of GadBH465A.

The fluorescence properties of wild type GadB, GadBH465A, and GadB\textsuperscript{HT} were analyzed at acidic and alkaline pH. When excited at 430 nm at pH 4.6 both wild type and mutants show an emission spectrum with a maximum at 500 nm (Fig. 6, A and B). The fluorescence changes significantly at alkaline pH; wild type GadB exhibits very little fluorescence upon excitation at 430 nm (Fig. 6A), whereas the fluorescence of both mutants increases and shifts to 522 nm (Fig. 6B).

Upon excitation at 345 nm the wild type enzyme and the mutants display different emission spectra both at acidic and alkaline pH. The emission spectrum of wild type GadB at pH 4.6 shows two maxima, at 390 and 500 nm, whereas at pH 7.5 it exhibits only one peak with a maximum at 390 nm (Fig. 6C). The emission spectrum at pH 4.6 of both His\textsuperscript{465} mutants displays a maximum at 390 nm, which decreases upon alkalization with a concomitant increase at 510 nm (Fig. 6D). Upon excitation of Trp residues at 295 nm (Fig. 6E), the emission spectrum of wild type GadB exhibits maxima at 355 and 490 nm at pH 4.6 and at 355 nm at pH 7.5. The fluorescence emission spectra of both mutants obtained upon excitation at 295 nm display maxima at 355 and 490 nm at acidic pH and at 355 and 520 nm at alkaline pH.

**pH-dependent Cellular Partition**—To assess whether in the two mutants the six N termini still form two triple helical bundles at acidic pH and whether this remains a cooperative process, despite the absence of cooperativity from the change in absorbance spectrum (Fig. 3C and Table 2), we relied on an earlier finding: the acid-induced formation of the triple bundles determines the partition of the enzyme between cytosolic and membrane fractions (4). The two fractions, obtained by ultracentrifugation of cell extracts from the E. coli strains overexpressing wild type GadB, GadBH465A, and GadB\textsuperscript{HT}, were resuspended at four different pH values: 5.5, 5.75, 6.0, and 6.2. The distribution of the different forms of the enzyme was determined by SDS gel electrophoresis and by activity measurements.
The proportion of all three proteins found in the membrane fractions increases with pH, and the process is cooperative. The transition midpoint is at pH 5.9 for all three proteins. The cooperativity levels are similar within the experimental error. However, for wild type enzyme the cooperativity is significantly lower than that governing the change in absorption spectrum (Fig. 3A, inset, and Table 2).

Catalytic Properties—Spectroscopic analysis provided evidence that in both His^{465} mutants the 420-nm absorbing species is still significantly present at very high pH values. This species is considered the catalytically competent one. The activity of both mutants was therefore assayed in the pH range 4.0–7.0 using a pH-stat device without added buffer (Fig. 8). Similar results were obtained by measuring the activity either in acetate or in phosphate buffer (Fig. 8, inset). The data of activity versus pH fit well to the Hill equation, suggesting that cooperativity still affects the enzyme activity (n = 2–3), although not at the same high levels as in the spectroscopic changes. The transition midpoint is at pH 5.9 for all three proteins. The cooperativity levels are similar within the experimental error. However, for wild type enzyme the cooperativity is significantly lower than that governing the change in absorption spectrum (Fig. 3A, inset, and Table 2).

TABLE 2
Hill parameters from curve fitting of the 420 nm absorbance readings as a function of pH

|        | n    | pK    |
|--------|------|-------|
| GadBH465A | 0.58 ± 0.06 | 7.43 ± 0.07 |
| GadB\Delta HT | 0.49 ± 0.06 | 7.55 ± 0.19 |
| GadB wt | 12.07 ± 1.82 | 5.308 ± 0.006 |
| GadB wt + NaCl | 6.74 ± 0.66 | 5.690 ± 0.007 |

The values reported were calculated using the integrated Hill equation.
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FIGURE 4. Deconvolution of spectra. A, absorption spectrum of wild type GadB at pH 6.84 resolved into its component absorption bands. The deconvolution of the spectrum of GadBH465A at pH 8.5 resolved into its component absorption bands. The deconvolution of the spectrum of GadB^ΔHT at pH 8.5 gave results identical to those for GadBH465A. The protein concentration was 7.7 μM. The spectra were recorded in 50 mM potassium phosphate buffers at the indicated pH. The solid lines represent the experimental spectra, the dashed lines are the component bands, and the dotted curve is the theoretical curve obtained by nonlinear least square fit of the experimental points to the sum of a variable number of log normal curves, each having independent parameters.

FIGURE 5. CD spectra at different pHs. The CD spectra of wild type GadB at pH 4.6 and 7.5 are shown with solid lines. The CD spectra of GadBH465A at pH 4.6 and 8.3 are shown with a dashed line. The CD spectra of GadB^ΔHT at pH 8.5 are omitted because they are identical to those of GadBH465A. Protein concentration was 60 μM as referred to the PLP content. The spectra were recorded in 50 mM potassium phosphate buffer at the indicated pH.

The production in bacteria of inducible decarboxylases was elegantly shown more than 60 years ago by Gale (27), who stated that it “may be the method by which the organism extends its (pH) range of existence.” Indeed the neutrophilic bacterium E. coli can survive for more than 2 h at pH ≤ 2.5, when glutamate is supplied in minimum medium and Gad activity is essential to this ability (28, 29). The acidic pH optimum of Gad is well suited to the intracellular pH 4.5, occurring upon exposure to an extremely acidic environment (pH < 2.5) like that of the stomach. Gad activity was suggested to be beneficial to the micro-organism by contributing to proton consumption and to the inversion of membrane potential, countering the entry of more protons (2, 4).

The present work was undertaken with the aim of providing new insights into the molecular basis of the pH-dependent mechanism of Gad regulation, in particular with respect to the role played by His^{465}, the residue locking the active site by forming an aldamine with the PLP-Lys^{276} Schiff base (3). One inevitable effect of mutating His^{465} is that the contribution of aldamine formation is eliminated from the pH-dependent equilibrium between active and inactive forms of the enzyme. A second inevitable effect is that the pH dependence of aldamine formation, which requires the imidazole of histidine to be deprotonated, is also eliminated. An unexpected finding was the major effect that the mutations exert on the cooperativity and midpoint of pH-dependent spectroscopic transition.

Cooperativity—Earlier experiments on GadB^Δ1–14 showed that the inability to form N-terminal helical bundles lowers the cooperativity from n >> 6 to n = 2 (3). Here we show that the formation of the triple helical bundles is still cooperative both in the His^{465} mutants and in wild type GadB, but in this case n = 3. The point of half transition is at pH 5.9. Notably O’Leary and Brummund (7) showed that E. coli Gad, both native and borohydride-reduced (in which aldamine formation/decomposition cannot occur), undergo a rate determining step, consisting of the loss of three protons, before detecting the spectral changes. Based on our and on previous results (3, 7), we suggest that the contribution from N-terminal helix formation to the cooperativity of the whole system is equivalent to n = 3 and that bundles fold/unfold independently from the events occurring at the active site. Thus the spectroscopic changes of GadB not only track the conformational changes in the active site but also indirectly record the rate-determining conformational changes at the distant N termini (3). It is tempting to assume that during evolution a histidine residue was selected for aldamine formation because the pK of its distal side chain nitrogen (6.0) is very close to the pK at which the transition between the folded and unfolded state of the bundles occurs, thus allowing both events (aldamine formation and bundle formation) to become linked.
paralogues. This kind of analysis detects which residues in a protein evolve neutrally and which ones, because of their structural or functional importance, are subjected to purifying selection (30). In the case of GadB the 14-residue-long C-terminal tail, although conformationally disordered at low pH, possesses one residue under strong purifying selection, and this is indeed that corresponding to *E. coli* GadB His465 (Fig. 10).

Because positive cooperativity in the spectroscopic transition is lost when His465 is removed (Fig. 3C), this residue is likely responsible for the residual cooperativity observed in GadBΔ1–14 (3, 4). Its absence massively affects the equilibrium between the open and closed active sites. This partially explains the finding that both mutants are at least twice as active as wild type GadB in the pH range 5.7–7.0 (Fig. 8).

In wild type GadB the level of cooperativity of the catalytic activity as a function of pH is significantly lower ($n = 2–3$) than that of the change in absorption spectrum. The difference likely arises because the absorption spectrum changes were observed on the free enzyme, whereas the activity measurements were initial velocities taken at high glutamate concentration. Under
the latter conditions, the active sites of the enzyme are fully occupied by glutamate so that His^{465} cannot enter. Notably a similar level of cooperativity, although not detectable at the spectroscopic level, is observed in the His^{465} mutants. Because the conformational change occurring at the N termini is still a cooperative process in the mutants, the cooperativity in the activity is likely due to one or more residues, also undergoing a conformational change. One candidate is Asp^{86}, a substrate-binding residue located on a loop taking up different conformations in the high and low pH forms of GadB (4).

The negative cooperativity of the spectroscopic changes in the mutants also needs to be explained. Based on the fitting of the experimental data, we assume that the pK of an ionizable group affecting the equilibrium between the 420- and the 334-nm absorbing species increases as a consequence of the conformational change. Such a perturbation in the pK of an ionizable group often occurs on catalytic groups of enzymes active sites and is known as the Born effect (or desolvation effect); it favors the neutral form of a titratable group when transferred from a polar to an apolar environment (31). Analysis of the His^{465} mutants revealed that an active site residue undergoes this change in pK when the active site environment of GadB becomes less polar.
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DCEB\textsubscript{ECOLI} : ******* : *******
DCEB\textsubscript{SHIFL} : ******* : *******
B3WXX\textsubscript{SHIDY} : ******* : *******
DCEA\textsubscript{ECOLI} : ******* : *******
B7LRF\textsubscript{ESC3F} : ******* : *******
B1EJ2\textsubscript{SESC} : ******* : *******
B7L9F\textsubscript{ESC3F} : ******* : *******
A1JZ6\textsubscript{EYER6} : ******* : *******
Q8GF15\textsubscript{EDWT} : ******* : *******
A2DC49\textsubscript{TRIVA} : ******* : *******
C0G9R8\textsubscript{HRHZ} : ******* : *******
B2UN99\textsubscript{AKM8} : ******* : *******
Q2K6V2\textsubscript{BORA1} : ******* : *******
A9WY34\textsubscript{BRUSI} : ******* : *******
A5VU56\textsubscript{BRUO2} : ******* : *******

**Figure 10.** Multiple sequence alignment of the C-terminal region of \textit{E. coli} GadB (UniProt code DCEB\textsubscript{ECOLI}) and 14 of its orthologues/paralogues. Background shading indicates residues under purifying selection in a $K_{d}/K_{a}$ ratio analysis of coding sequences (the darker the shading the stronger the purifying selection, light gray corresponds to SELECTON score 5, dark gray corresponds to 6, and black corresponds to 7). A black square denotes the last residue (Pro\textsuperscript{466}) of the folded core of GadB, after which the 14-residue-long C-terminal tail begins. The other sequences in the alignment are from: \textit{S. flexneri} (DCEB\textsubscript{SHIFL}), \textit{Shigella dysenteriae} 1012 (B3WXX\textsubscript{SHIDY}), \textit{E. coli} GadA (DCEA\textsubscript{ECOLI}), \textit{Escherichia fergusonii} (B7L9F\textsubscript{ESC3F}), \textit{Escherichia coli} (1EJ2\textsubscript{SESC}), \textit{E. fergusonii} GadA (B7LP95\textsubscript{ESC53}), \textit{Yersinia enterocolitica} strain O8 (A1JZ6\textsubscript{EYER6}), \textit{E. coli} (C0G9R8\textsubscript{HRHZ}), \textit{Acinetobacter baumannii} TW027 GadA (B1EJ2\textsubscript{SESC}), \textit{E. fergusonii} GadA (B7LP95\textsubscript{ESC53}), \textit{Yersinia enterocolitica} strain O8 (A1JZ6\textsubscript{EYER6}), \textit{E. coli} (C0G9R8\textsubscript{HRHZ}), \textit{Escherichia coli} (A2DC49\textsubscript{TRIVA}), \textit{Brucella ceti} strain Cudo (C0G9R8\textsubscript{HRHZ}), \textit{Escherichia coli} (B2UN99\textsubscript{AKM8}), \textit{Brucella suis} (Q2K6V2\textsubscript{BORA1}), \textit{Brucella suis} (A9WY34\textsubscript{BRUSI}), and \textit{Brucella suis} (A5VU56\textsubscript{BRUO2}).

**Spectroscopic Properties—** \textit{E. coli} GadB is the only PLP-dependent enzyme where an aldamine form of the cofactor was crystallographically observed (3). Thus it can be used to analyze the aldamine contribution to the spectroscopic properties of a PLP-dependent enzyme with those of chemical models (9, 15). UV-visible spectroscopy confirms that in the absence of His\textsuperscript{465}, the active site of GadB is less efficiently locked. This is almost undetectable optical activity, confirming the hypothesis of O'Leary and Brummund (7). A model PLP-hexylamine Schiff base is being detected in these conditions. In model studies the fluorescence emission of enolimine is characterized by an unusually high Stoke shift ($9,000–11,000$ cm$^{-1}$) (15) because of an intramolecular proton transfer in the excited state, yielding the ketoenamine as the excited species, which then emits fluorescence at long wavelengths (15). The rates of proton transfer and radiative decay from the ketoenamine ($\approx$510 nm) or directly from the enolimine ($\approx$430 nm) were suggested to account for the relative intensities of the two fluorescence emission maxima in phosphorlyase $b$ and other PLP-dependent enzymes (33, 34). Thus we suggest that in the absence of His\textsuperscript{465} side chain the enolimine tautomter is present in the active site of GadB; enolimine is typically detected when the active site becomes less polar (9). This is in accord with previous findings with the GadB $\text{Lys}^{276}$ mutant (5). The complexity of the fluorescence emission spectrum of the 330–340-nm absorbing species in GadB appears to arise from the combined effects of several factors: the C4’ hybridization, the radiation energy-induced intramolecular proton transfer between Schiff base tautomers, and their respective radiation decay rates. However, based on the present findings, it cannot be excluded that similar results could be obtained with carbaminol (34).

**Catalytic Properties—** It has been recently shown that wild type GadB can be efficiently entrapped in calcium alginate beads (immobilized) and used in a reactor set-up with a pH-stat to perform the decarboxylation reaction at pH 4.6 in the absence of a buffer system (20). Immobilization does not affect the pH dependence of enzyme activity. Because glutamic acid is abundant in waste streams from biofuel production, it is regarded as an interesting starting material for the synthesis of nitrogen-containing bulk chemicals, which can be derived from GABA (20). Because in GadB\textsubscript{H465A} and GadB\textsubscript{H465E} the active site “lock” cannot be formed and the range of activity is significantly extended toward alkaline pH, they are very attractive for the above application. Indeed at pH 5.9 the specific activity of both mutants is four times higher than that of wild type GadB. At this pH glutamic acid is much more soluble than at pH 4.6 (35). This is very advantageous for use in a bioreactor, because it removes, with massive gains in efficiency, the bottleneck caused by the limited solubility of glutamate at the acidic pH optimum of wild type GadB.

In conclusion, aldamine formation contributes significantly to the inactivation of GadB and to its pH-dependent activity profile. The $K_{d}$ of the distal imidazolic nitrogen of His\textsuperscript{465} affects the pH-dependent spectroscopic and catalytic properties of \textit{E. coli} GadB, and His\textsuperscript{465} must be present to integrate the cooperativity of triple helix formation into the cooperativity of the whole system. Notably, in the eukaryotic relative of \textit{E. coli} GadB, \textit{Arabidopsis thaliana} Gad1, this residue is not found in the long C-terminal tail, instrumental to activity regulation by pH and by Ca$^{2+}$/calmodulin binding, and this probably explains why Gad1 does not undergo the same mechanism of inactivation (19).
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