The Roles of His-167 and His-275 in the Reaction Catalyzed by Glutamate Decarboxylase from *Escherichia coli*  

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Two histidine residues in glutamate decarboxylase from *Escherichia coli*, potential participants in catalysis because they are conserved among amino acid decarboxylases and because they are at the active site in the homologous enzyme ornithine decarboxylase, were mutated. His-275 is shown to bind the cofactor pyridoxal 5'-phosphate but not to contribute directly to catalysis. The H275N enzyme was unable to bind the cofactor whereas the H275Q mutant contained 50% of the normal complement of cofactor and its specific activity (expressed per mole of cofactor) was 70% of that of the wild-type enzyme. The H167N mutant bound the cofactor tightly, its specific activity was approximately half that of the wild-type enzyme and experiments in D2O showed that it catalyzed replacement of the carboxyl group with retention of configuration as does the wild-type enzyme. Comparison of reaction profiles by observing changes in the absorbance of the cofactor after stopped-flow mixing, revealed that a slow reaction, in which approximately one-third of the wild-type enzyme is converted to an unreactive complex during catalysis, does not occur with the H167N mutant enzyme. This reaction is attributed to a substrate-induced conformational change, a proposal that is supported by differential scanning calorimetry.

Glutamate decarboxylase is a member of a large family of pyridoxal-phosphate (PLP)<sup>1</sup>-dependent enzymes which catalyze a wide variety of different reactions on their amino acid substrates (1). Although the enzyme from *Escherichia coli* is the most studied of the amino acid decarboxylases, its three-dimensional structure has not been solved. However, sequence comparisons show that it is homologous to ornithine decarboxylase from *Lactobacillus 30a*, the three-dimensional structure of which (2, 3) shows two histidine residues, His-223 and His-354, close to and on either side of the cofactor (2, 3). These histidines are conserved in the sequences of most, if not all, of the PLP-dependent amino acid decarboxylases (2). There is no doubt that His-275 of *E. coli* glutamate decarboxylase aligns with His-354 of ornithine decarboxylase since, in both enzymes as in other amino acid decarboxylases, it immediately precedes the lysine residue that forms an imine with the coenzyme (Fig. 1). In ornithine decarboxylase, the imidazole ring of His-354 contributes to binding the 5'-phosphate of the cofactor and is considered unlikely to participate in catalysis unless it is displaced by a substrate-induced conformational change (2, 3). Alignment of His-167 of glutamate decarboxylase is less clear because sequence similarity between the two enzymes is weak in this region (Fig. 1). However, the presence, in ornithine decarboxylase, of the imidazole ring of His-223 just below the imine of the cofactor on the face, suggests that this arrangement may be a common feature of the amino acid decarboxylases. His-167 in glutamate decarboxylase is the only histidine residue that can reasonably occupy this position unless there are major differences between the folds of the two proteins.

Several stages in the catalytic mechanism of amino acid decarboxylation involve proton transfers for which histidine residues might be responsible (Scheme 1). The formation of an external aldimine (III) requires multiple proton transfers and, after decarboxylation to give the quinonoid intermediate (IV), the carboxyl group is replaced by a proton. Further proton transfers, analogous to those occurring in external aldime formation, are required for liberation of the product 4-aminobutyrate. An additional protonation occurs in a side reaction where a proton is added to C4 of the cofactor rather than Cα of the substrate to give an external aldime of pyridoxamine phosphate with succinic semialdehyde. This reaction, which occurs only once in 3 × 10<sup>5</sup> turnovers in *E. coli* glutamate decarboxylase (4), is a feature of most amino acid decarboxylases (5–9). In some of these enzymes this abortive transamination is kinetically more prominent and is almost certainly metabolically important because it inactivates the enzyme by leaving the cofactor as pyridoxamine phosphate.

Considerable attention has been given to the stereochemistry of amino acid decarboxylation and subsequent protonation. Decarboxylation requires the carboxyl group to be orthogonal to the plane comprising the cofactor pyridinium ring and the imine double bond (10). The proton which replaces the carboxyl group at Cα arrives from the same direction in which the carboxyl group has left (11). Retention of configuration has also been observed in all other amino acid decarboxylases in which the question has been investigated (12–14). Kinetic evidence (15) indicates that, in methionine decarboxylase, a histidine residue protonates at Cα, whereas a lysine residue protonates at C4 and it has been argued that lysine and histidine perform the corresponding protonations in glutamate decarboxylase (16). In the present work, we have examined the effect of mutations at His-167 and at His-275 on the kinetic and structural properties of the enzyme.

**EXPERIMENTAL PROCEDURES**

Analytical Reagents—4-Aminobutyrate aminotransferase was prepared as described (17). D<sub>2</sub>O was from Sigma, Vent polymerase was
Histidin of Glutamate Decarboxylase

**Fig. 1.** Sequence alignment of Lactobacillus 30a ornithine decarboxylase (ODC) and E. coli glutamate decarboxylase (GAD).

The alignment is that given in Ref. 2 with glutamate decarboxylase residues 155–177 displaced by one residue toward the amino terminus. Conserved residues are boxed, the mutated histidine residues are in bold, and the active site lysine is marked by an asterisk.

from New England Biolabs. Restriction enzymes, T4 DNA ligase, the agarose gel DNA extraction kit, and Gabase were from Boehringer. The T7 sequencing kit, DEAE-Sepharose, and Sephadex G-25 were from Pharmacia. [γ-35S]dATP (1000 Ci/mmol) was from NEN Life Science Products. Ingredients for bacterial growth were from Difco. Oligonucleotides were from Genencor. Other chemicals were from BDH.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed by overlap extension polymerase chain reactions (18). External primers annealing over the N- and C-terminal sequences were those used in the construction of the expression plasmid containing the *gadB* gene (19). Mutagenic primers for H167N were 5'-CTTCTGGAATAATTCG-3' and their complementary sequence. Those for H275N and H275Q were 5'-GCTTCAGGCCAGAAATTCG-3' and 5'-GCTCTAGAAGGCTAACCG-3' and their complementary sequences, respectively.

Plasmid pQoQaB was used as template. The products of polymerase chain reactions (25 cycles), carried out with 2.5 units of Vent polymerase with denaturation at 95 °C for 1 min, annealing at 45 °C (H167N) or 48 °C (H275N and H275Q) for 1 min, and extension at 74 °C for 2 min, were used as templates with the external primers to generate the complete coding sequence of glutamate decarboxylase. Fragments (NovElEcoRV for H167N and EcoRV/HindIII for H275N and H275Q) were subcloned into pQoQaB. The newly inserted parts of the expression construct, pQoQaH167N, pQoQaH275N, and pQoQaH275Q, were sequenced and the plasmids were used to transform E. coli JM109 carrying the plasmid pREP4. Expression, purification, and assay of mutational forms of glutamate decarboxylase were as described for the wild-type enzyme (19) except where stated.

Reconstitution of Apo-enzyme with N-(5'-Phosphopyridoxyl)glutamate and Calorimetric Analysis—N-(5'-Phosphopyridoxyl)glutamate was prepared by treating 0.5 mM sodium glutamate and 0.5 mM PLP (pH 4.5) with sodium cyanoborohydride (10 mM). Apo-forms of wild-type and mutant forms of glutamate decarboxylase (10 mg/ml in 0.1 M piperazine-acetate, pH 6.8) were reconstituted with a 5-fold molar excess of N-(5'-phosphopyridoxyl)glutamate for 1 h (25 °C). Samples were concentrated, separated on Sephacryl G-25, and the proteins dialyzed against 20 mM sodium acetate (pH 3.6) containing 0.1 mM dithiothreitol. Thermal unfolding of deagassed samples (1.5–2.0 mg/ml) was analyzed under nitrogen pressure on a MicroCal MC-2D calorimeter (MicroCal, Inc., Northampton, MA). Results were corrected for instrumental baseline and normalized for protein concentration. No reversibility was observed in a second heating cycle.

**Deuterium Exchange Reactions**—Enzyme samples were brought into 99.5% D2O by repeated concentration and dilution. The enzyme (0.8 μM) was mixed with glutamate (14.25 mM), DCI was added to maintain constant pD of 4.6 (reading on pD meter = 4.2). At the end of the reaction (2 h) the solution was neutralized by adding solid Tri, lyophilized, and redissolved in 0.5 ml of D2O. NMR spectra were determined using a Bruker AMX 360 spectrometer. The stereochemistry of the deuterated 4-amino-butyrate was determined by repeating the NMR analysis after an overnight incubation in the presence of 4-amino-butyrate in the absence (0.26 mM) and presence (0.26 mM) of enzyme.

**Stopped and Quenched Flow Measurements, Spectrophotometry**—Stopped-flow experiments were performed on a SF-1 stopped-flow spectrophotometer (Hi-Tech, Salisbury, United Kingdom). Product formation during the period from 0.2 to 7 s was measured using a quenched flow apparatus (8, 20). Reactions lasting longer than 7 s were stopped manually. Curve fitting and statistical analyses were performed using the data manipulation software Scientist (Micromath, Salt Lake City, UT). Absorption spectra were measured with a Hewlett-Packard model 8452 diode-array spectrophotometer. CD spectra were recorded as the average of 3 scans on a Jasco 710 spectropolarimeter equipped with a DP 520 processor at 25 °C using a 2-mm quartz cell.

**RESULTS**

**Stability, Specific Activity, Cofactor Content, and pH Dependence of Absorption Spectra**—The far UV CD spectra of the H167N and H275Q mutants were identical with that of the wild-type enzyme (Fig. 2) indicating that these mutations had not introduced major changes in the global structure of the enzyme. The H275N mutant precipitated too rapidly at low concentrations to permit far UV CD analysis. Yields of the H167N mutant enzyme after the standard purification, which does not include added PLP, were as high as those of the wild-type enzyme. This form, like the wild-type enzyme, was stable for many months at 4 °C and for several hours at room temperature. Its absorption and CD spectra (Fig. 2) were almost identical with those of the wild-type enzyme, indicating that one molecule of cofactor was bound per monomer. The specific activity (126 μmol min⁻¹ mg⁻¹) was approximately half that of the wild-type enzyme. Differential scanning calorimetry of the wild-type enzyme (Fig. 3) showed that the transition temperature of the apoenzyme reconstituted with the cofactor substrate-cocofactor adduct, N-(5'-phosphopyridoxyl)-glutamate, was 8 °C higher than that of the native holo-enzyme (51 °C) suggesting that binding of glutamate at the active site stabilizes the structure considerably. When the same experiment was carried out on the H167N enzyme, the holoenzyme was found to be 4 °C more stable than the wild-type enzyme but the mutant was not further stabilized when the cofactor was replaced by N-(5'-phosphopyridoxyl)glutamate (Fig. 3).

Purification of the H275N enzyme according to the standard protocol resulted in a yield of enzyme protein 63% of that normally achieved with the wild-type enzyme. The absorption spectrum showed that this form contained no cofactor. Inclusion of PLP (0.1 mM) in solutions used for dialysis gave an enzyme preparation that clearly bound the cofactor but with an absorption spectrum (Fig. 2a) which showed more 340 nm chromophore than is present in the wild-type enzyme. The instability of this form of the enzyme prevented measurement of its kinetic properties.

The H275Q mutant was more stable, although, after the standard purification, its absorption spectrum (Fig. 2a) showed that its coenzyme content was lower than that of the wild-type enzyme. Treatment with 0.1 M NaOH to release the PLP showed it to contain 0.5 mol of cofactor per mol of subunit. Its specific activity was 80 units/mg (35% that of the wild-type enzyme). Thus, per mole of cofactor, the specific activity of this mutant is 70% that of the wild-type enzyme.

Glutamate decarboxylase undergoes a sharp, pH-dependent, transition in which a 420-nm absorbing form, presumed to be the protonated internal aldime, is converted to a 540-nm form. The transition involves uptake of multiple protons and lasts several seconds, demonstrating that a slow, protonation-dependent reaction is involved as well as the protonation itself (21, 22). The midpoint of the transition (pH 5.5) is within the range expected for protonation of a histidine imidazole. Comparison of absorption spectra of the wild-type enzyme at different pH values with those of the H167N and H275Q mutants showed that both mutants behaved as the wild-type, demonstrating that neither His-167 nor His-275 is responsible for the pH-dependent transition.

**Deuterium Exchange Reactions**—In ornithine decarboxylase,
His-223 which we hypothesize to be equivalent to His-167 in glutamate decarboxylase, occupies a position on the re face of the cofactor. Lys-355 in ornithine decarboxylase is on the si face and the equivalent residue in glutamate decarboxylase is Lys-276. In the aminotransferases from the same family, the proton transfers occur on the si face and are mediated by the equivalent of Lys-276 (23). It is known that decarboxylation of glutamate occurs with retention of configuration (11) but it is not known from which side the carboxyl group leaves. Quantum mechanical calculations (4) confirm that the CO-COO⁻ bond has two positions of maximal lability, each perpendicular to the coenzyme ring as predicted by Dunathan (10), but pointing in opposite directions. It is possible therefore that the carboxyl group leaves from the re face and that protonation of Ca is mediated from this face by His-167. To test this possibility, the 4-aminobutyrate produced by wild-type and H167N glutamate decarboxylase in D₂O was analyzed by NMR. In both cases the signal from protons at C₄ was halved, indicating that the 4-aminobutyrate produced by each form of the enzyme was monodeuterated at C₄ (equivalent to C₁ of glutamate). When the products were treated in D₂O with 4-aminobutyrate aminotransferase, which labilizes the proton at C₄ of glutamate exclusively (24), the signal from C₄ protons was lost in each case. This indicated incorporation of a second deuterium at C₄.

Rapid Mixing and Quenching Experiments—Significant differences between the H167N mutant and the wild-type enzyme were observed when changes in the absorption of the cofactor were measured after stopped-flow mixing with glutamate.

The reaction with the most readily interpreted kinetic behavior was that of the H167N mutant. Changes in absorbance were largest at 322 nm. A large increase, complete within the mixing time, was followed by an initial decrease which followed a single exponential (Fig. 4a, k = 30 ± 1 s⁻¹). Thereafter, absorbance fell as expected for a system in which the concentration of ES complex returns to zero as substrate is consumed in the reaction (Fig. 4b). A single exponential of this kind, in which the pseudo-equilibrium mixture of enzyme-substrate complexes is observed throughout the full course of the reaction from [S] ≫ Kₘ to [S] = 0, provides an accurate estimate of the steady state constants kₐₚ and Kₘ (The system is not subject to product inhibition since the kinetic constants were independent of initial substrate concentration and inclusion of 4-aminobutyrate had no effect.) After exclusion of the first 0.2 s because of the fast pre-steady state reaction, the profile gave an excellent fit to the Michaelis equation and provided values for the steady state constants kₐₚ = 27.8 ± 0.1 s⁻¹; Kₘ = 10.6 ± 0.1 mM. The similarity of the values obtained for kₐₚ and the rate constant characterizing the pre-steady state transient, suggests that the reaction reported by the latter is largely rate-determining in the overall process. At 420 nm the absorbance changes observed were much smaller (total absorbance change 0.02) and in the opposite direction but they were governed by the same rate constants. Because of the unusual kinetic behavior of the wild-type enzyme (see later), product formed during the first 10 s of reaction was measured using the quenched-flow apparatus. Conditions for the enzyme-catalyzed reaction were exactly as in the stopped-flow experiment. Fig. 4c shows the result. The line of best fit through the experimental points from the quenched flow experiment is 0.98 mM s⁻¹. The values of kₐₚ and Kₘ determined from the stopped flow experiment predict 0.93 mM s⁻¹.

The absorbance changes observed with the wild-type enzyme were more complex (Fig. 5a). Large changes were observed at 322, 360, and 420 nm. At 322 and 360 nm an initial increase was complete within the mixing time. This was followed by an increase lasting approximately 5 s. After exclusion of the first 5 s the reaction showed A₃22 and A₃60 declining as expected for a system which is monitoring ES complex as substrate is consumed. A fit to the Michaelis equation gave values of kₐₚ = 55 s⁻¹ and Kₘ = 6.7 mM. At 420 nm, changes were in the opposite direction but were characterized by the same rate constants. Inclusion of the product 4-aminobutyrate (50 mM) did not affect the kinetic profile, confirming earlier observations that product inhibition is insignificant (5). The complete kinetic profile was fitted, by nonlinear regression using differential equations, to a scheme in which, after initial rapid reaction to form a pseudo-equilibrium mixture of complexes (ES), a species (EX) is formed which does not lie on the path leading to product formation (Scheme 2).

\[
\begin{align*}
E + S & \rightarrow ES \\
& \rightarrow E + P \\
& \text{Scheme 2}
\end{align*}
\]

The data (Fig. 5a) fit well to Scheme 2 and give kinetic constants of k₁ = 0.32 ± 0.02 s⁻¹, k₂ = 0.4 ± 0.1 s⁻¹, kₑ = 98 ±
coefficients to the species $E$ determined in 50 mM sodium acetate (pH 4.6), using $50 \mu M$ enzyme. High wavelength CD spectra (b, inset) were determined in 50 mM sodium acetate (pH 4.6), using 50 $\mu M$ of each form of the enzyme (coenzyme concentration). In this case the spectrum of the wild-type enzyme is omitted because it is identical with that of H167N. The reference solutions against which the spectra were measured were those used for dialysis at the final stage of preparation.

The absence of the transient phase from the H167N mutant enzyme is the most striking difference between this mutant and the wild-type enzyme. Thus the nature of the chromophore formed in the process is important in understanding the role played by His-167. The approximately equal values of $k_1$ and $k_2$, and the approximately 2-fold difference between $k_{cat}$ and $k_{cat}$ suggest that, during the first few seconds of reaction, approximately half of the enzyme converts into a form which cannot react further. Such a process should therefore affect the rate of product formation and result in a burst at the beginning of the reaction. A quenched-flow apparatus was therefore used to measure the formation of 4-aminobutyrate during the first 15 s of reaction. The results (Fig. 5a, inset) show a clear burst which fits well to the same rate constants and confirms that formation of the 345-nm chromophore is effectively inhibitory.

**Solvent Isotope Effects**—During the investigations aimed at measuring the incorporation of deuterium into the product 4-aminobutyrate (see “Deuterium Exchange Reactions”), we noticed large differences in the rates at which acid needed to be added to maintain constant pH in the reactions catalyzed by the wild-type enzyme. An earlier study of the effects of $D_2O$ on the reaction catalyzed by glutamate decarboxylase has already shown large solvent isotope effects (25) and these have been cited in support of a proposal that, after the decarboxylation step, protonation at Ca is mediated by a histidine residue (16). To investigate these effects further, we conducted rapid mixing experiments in $D_2O$ with wild-type enzyme and with the H167N mutant. We measured the steady state constants by analyzing the complete reaction profile after omitting the first 5 s containing the transient. Our results for the wild-type enzyme (Fig. 5b) are in broad agreement with those published earlier (25). We found $k_{cat}$ (26.3 ± 0.8 s$^{-1}$) to be 2.7 times smaller and $k_{cat}/K_m$ (2.3 ± 0.1 mm$^{-1}$ s$^{-1}$) to be 3.5 times smaller than in $H_2O$. We also observed a pronounced solvent isotope effect on the slow transient, the amplitude of which was greatly increased both at 420 and 322 nm. The whole process, including the slow transient, fitted well to Scheme 2 and gave constants of best fit $k_1 = 0.84 ± 0.37$ s$^{-1}$, $k_2 = 0.4 ± 0.1$ s$^{-1}$, $k_3 = 74 ± 38$ s$^{-1}$, and $K = 32 ± 12$ mm. The fit assigned extinction coefficients to the species $E$ and $EX$ of $2748 ± 58$ m$^{-1}$ cm$^{-1}$ and $4290 ± 810$ m$^{-1}$ cm$^{-1}$, respectively. The in-
creased size of the transient suggested that, if it is due to slow formation of a species off the reaction pathway, the course of product formation should be characterized by a more pronounced burst when the reaction is carried out in D$_2$O. Fig. 5b (inset) shows the course of 4-aminobutyrate production measured by quenched-flow over the first 10 s of reaction and confirms that a pronounced burst is present. The constants of best fit for these data were $k_1 = 2.3 \pm 1.7$ s$^{-1}$, $k_2 = 0.65 \pm 0.15$ s$^{-1}$, $k_3 = 42 \pm 19$ s$^{-1}$, $K_m = 32 \pm 12$ mM. Although the constants derived from the different types of experiment are not as closely similar as those from the corresponding experiments in H$_2$O, we consider that, in view of the large standard deviations, Scheme 2 also provides a satisfactory explanation.

When the H167N mutant enzyme was used to catalyze the deuterium exchange reaction, the amount of acid required to maintain constant pH in unbuffered solution in D$_2$O or H$_2$O was the same. The reaction profile monitored at 322 nm after stopped-flow mixing was also similar to that obtained in H$_2$O but analysis showed that $k_{cat}$ (38.2 ± 0.1 s$^{-1}$) was increased 1.4-fold and $k_{cat}/K_m$ (1.66 mM$^{-1}$ s$^{-1}$) was decreased 1.6-fold. The slow conversion to an unreactive intermediate does not occur with this form of the enzyme so that this complication is eliminated as a contribution to the solvent isotope effects. We note that, in the absence of the side reaction, a positive isotope effect on $k_{cat}$ was observed rather than the larger negative effect seen in the reaction of the wild-type enzyme. The increased amplitude of the slow transient phase in D$_2$O and the accompanying more marked nature of the burst in product formation strengthen the evidence that an unreactive complex is formed in this phase.

Decarboxylation-dependent Transamination—The 345-nm chromophore formed in the slow side reaction has been proposed (4) to arise by tautomerization of the quinonoid structure formed after decarboxylation (Scheme 1; IV). In this proposal, the proton on N of the cofactor-substrate imine has transferred to O3’ of the cofactor to give an intermediate which was considered to be more likely to protonate at C4’ than at C6 and thereby to lead to the abortive transamination reaction. The H167N mutant provides a test of this proposal because it clearly does not undergo the proposed tautomerization and should therefore not lose activity progressively by transamination. The transamination reaction was measured experimentally (4) by following the course of product formation over a long period (100 min). Despite the fact that substrate concentration remained high, the reaction slowed and stopped but was restarted by addition of PLP. We have confirmed these observations and additionally found that the H167N mutant shows the same behavior (Fig. 6). It is clear that the rate of inactivation through transamination is the same when His-167 is absent even though the 345-nm chromophore is not formed. Therefore the formation of the 345-nm chromophore is not required as a precursor to transamination and its identification as the tautomer of the quinonoid (4) is probably incorrect. It was noticeable that, using the conditions described in Ref. 4 (pyridine HCl as buffer), the rate of the reactions catalyzed by the H167N mutant was only about 30% less than that of the wild-type enzyme compared with 50% in acetate buffer.

**DISCUSSION**

The experiments with glutamate decarboxylase mutated at His-275 show that this residue contributes significantly to binding the cofactor and that glutamine is an adequate replacement whereas asparagine is not. Because the amide nitrogens of glutamine and asparagine superimpose on the τ and π nitrogens of histidine, respectively, it is proposed (26) that substitution of histidine by these two residues allows identification of the imidazole nitrogen involved in a functional hydrogen bond. Our observations indicate that the τ nitrogen of His-275 makes a hydrogen bond with the cofactor. The corresponding histidine (His-231) in PLP-dependent histidine decarboxylase has also been replaced either by asparagine or glutamine (27). Estimates of the activity of these mutants in crude extracts showed that the glutamine mutant had 12% of the specific activity of the wild-type enzyme whereas the asparagine mutant was barely active (0.16%). The authors considered inefficient coenzyme binding to be the cause of the lower activity and the τ nitrogen was identified as the atom responsible for hydrogen bonding (27). This also appears to be the arrangement...
Histidines of Glutamate Decarboxylase

Fig. 5. Changes in cofactor absorbance and product concentration during the reaction catalyzed by wild-type glutamate decarboxylase. Reactions were conducted (a) in H$_2$O and (b) in D$_2$O. Conditions as in Fig. 4. The solid lines are those of best fit to Scheme 2 using the constants given in the text. Only one in 10 of the data points collected and used in fitting is shown so that the fit of experimental points to the theoretical lines can be seen.

in the crystallographic structure of ornithine decarboxylase where His-354 makes a hydrogen bond with an oxygen of the cofactor 5'-phosphate (3). The evidence thus suggests that a hydrogen bond between the cofactor phosphate and the histidine that almost invariably precedes the cofactor-binding lysine in the sequence, is a common feature in the family of amino acid decarboxylases.

The similarity of the values obtained from analyzing the complete reaction profile of the H275Q mutant to those obtained for the wild-type enzyme agrees well with the observation that this form of the enzyme has a specific activity 70% that of the wild-type enzyme. We conclude that His-275 is not essential for catalyzing any of the proton transfers in the reaction. This conclusion is to be expected if, as argued above, His-275 occupies a position equivalent to that of the corresponding histidine in ornithine decarboxylase (3) where the nitrogen of the imidazole ring are too far from C9 of the substrate to act as proton donor.

The fact that His-275 can be replaced by glutamine without serious loss of the ability to bind the coenzyme or to catalyze the reaction, raises the question of why it is so strongly conserved within the amino acid decarboxylases. In the evolutionarily-related B6 enzyme, serine hydroxymethyltransferase, it can be replaced by asparagine without substantial loss of activity or ability to bind the cofactor (28). It was suggested that in this enzyme it interacts with the substrate to induce a closure of the enzyme necessary for specificity (28). The enzyme-binding lysine in tryptophan synthase, which belongs to a completely different family, is also preceded by a histidine which can be mutated without major loss of enzyme activity or ability to bind the cofactor (29). In this case the structure shows the imidazole to be near the cofactor phosphate but not as near as would be expected for bonding. This histidine is not present in any of the 51 sequences of 14 different aminotransferases even though some of these are undoubtedly evolutionarily related to the group of amino acid decarboxylases of which E. coli glutamate decarboxylase is a member (1). In many of the aminotransferases, the corresponding residue is serine and in E. coli aspartate aminotransferase, for which a structure is available, this serine makes a hydrogen bond with the 5'-phosphate of the cofactor just as we propose for the histidine of glutamate decarboxylase. The fact that preparations of the H275Q glutamate decarboxylase mutant have less than the full complement of cofactor shows that glutamine is not a perfect replacement so that substitution of the histidine incurs a penalty which may have been sufficient to ensure that this residue was conserved.

Our hypothesis that His-167 functions as the general acid that protonates at C9 was constructed on the basis that this residue is not responsible for protonating C9 of the substrate to act as proton donor.

The hypothesis was clearly wrong. Comparison of specific activities and of $k_{cat}$ and $k_{cat}/K_{m}$ values showed that the H167N mutant was only 2–3-fold less active than the wild-type enzyme. Thus His-167 does not contribute greatly to any rate-limiting protonation in the mechanism. However, on this evidence alone, it remained possible that His-167 ensured very rapid protonation from the re side. In this case, substitution of His-167 could yield a form of the enzyme which still catalyzes efficient decarboxylation but without the stereochemical fidelity of the wild-type enzyme. The deuterium exchange experiments demonstrate that the direction in which the carboxyl group is replaced is unaffected by mutation of His-167, showing that this residue is not responsible for protonating at C9 of the substrate. We conclude that His-167 is not directly involved in catalysis.
However, His-167 does play a significant part in events that occur after glutamate has bound. The slow conversion of the wild-type enzyme to an unreactive complex during the first few seconds of reaction, is not detectable in the H167N mutant. The absorbance changes that the cofactor undergoes in the first few seconds after mixing with glutamate have been reported earlier (21, 4) and it has been shown that the chromophore formed absorbs maximally at 345 nm (4). However, the kinetic constants characterizing the different phases were not determined and it was not shown that the process leads to a less active form of the enzyme. In Scheme 2, the complex EX lies off the path leading to product. Its formation is analogous to the slow appearance of uncompetitive inhibition. \( k_m \) and \( k_{cat} \) are decreased in the same proportion by its existence and \( k_{cat}/K_m \) is therefore unchanged. Thus, when \([S] \ll [E] \), the formation of this unreactive complex will not result in significantly lower steady-state rates. If, as is common for most enzymes, glutamate decarboxylase normally operates far from saturation, the side reaction will not be metabolically disadvantageous.

Some deductions can be made about the nature of the side reaction promoted by His-167. Immediately after mixing, the system has high absorbance at 420 nm. At the substrate concentrations used, the enzyme is almost saturated so that the intermediate at highest concentration must be one of the two external aldmines, either that with glutamate (III in Scheme 2) or that with the product 4-aminobutyrate. The most reasonable explanation for the large difference in coenzyme spectrum from a 420-nm absorbing chromophore to one that absorbs at 345 nm is that a proton has been lost from the external aldmine, either dissociated altogether or transferred to O3’ to give a less resonant tautomer. However, the process is far too slow to be due directly to proton transfer and it seems likely that it results from a slow conformational change, induced by the binding of glutamate, to a form with a lower \( pK_a \) for the external aldmine or a lower polarity favoring the less polar tautomer. The calorimetric evidence (Fig. 3) supports this proposal because it shows that the binding of glutamate stabilizes the wild-type enzyme but not the H167N mutant. The fact that the transition temperature is already 4 °C higher in the H167N holoenzyme indicates that this mutant enzyme has probably already adopted at least part of the conformational change normally induced by substrate. Effects of this kind are seen with other PLP-dependent enzymes which are known to undergo substrate-induced conformational changes (30, 31).

The absence of the side reaction in decarboxylation catalyzed by the H167N mutant shows that most of the previously observed and unexpectedly large solvent isotope effect on \( k_{cat} \) (25) is due to the increase in the extent to which the unproductive side reaction occurs and not to an effect on a protonation that is directly involved in the chemical transformations. This observation illustrates the difficulties associated with interpreting solvent isotope effects in enzyme-catalyzed reactions.

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