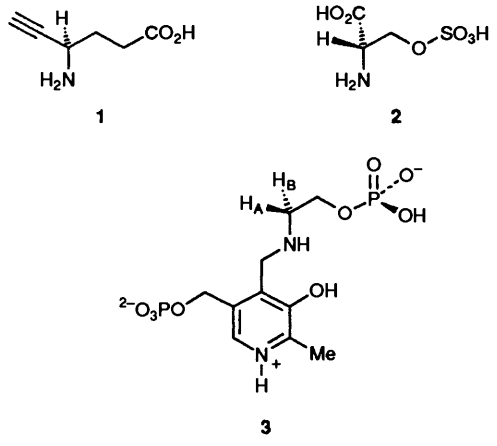
The Stereochemical Course of Decarboxylation, Transamination and Elimination Reactions Catalysed by *Escherichia coli* Glutamic Acid Decarboxylase[1]

Kevin Tilley,[a] Mahmoud Akhtar[b] and David Gani*[,b]
[a] *Chemistry Department, University of Southampton, Southampton SO9 5NH, UK*
[b] *School of Chemistry, The Purdie Building, The University, St. Andrews, Fife KY16 9ST, UK*

Pyridoxal 5'-phosphate dependent *Escherichia coli* glutamic acid decarboxylase reprotonates the quinonoid intermediate derived from the coenzyme and its natural substrate, (2*S*)-glutamic acid on the 4'-*Si*-face of the coenzyme during an abortive decarboxylation–transamination reaction. The enzyme introduces the 3-*pro-R* hydrogen of β-alanine with retention of configuration during the decarboxylation of (2*S*)-aspartic acid. In the absence of pyridoxal 5'-phosphate, treatment of the inactive apoenzyme with the inhibitor $N^{4'}$-(2''-phosphoethyl)pyridoxamine 5'-phosphate results in reactivation through the formation of the active pyridoxal 5'-phosphate holoenzyme complex. During this reaction hydrogen phosphate is eliminated from the phosphoethyl moiety. Using synthetic chirally deuteriated isotopomers of the inhibitor it is demonstrated that the 1-*pro-R* hydrogen of inhibitor is removed during the reactivation reaction. The results suggest that protonations and deprotonations at C^{α} of quinonoid intermediates derived from the coenzyme and the substrate occur from the 4'-*Si*-face of the coenzyme and that the distal binding groups of the substrates and inhibitors occupy similar positions at the active site on the 3'-phenolic group side of the coenzyme.

Pyridoxal 5'-phosphate (PLP) dependent decarboxylases are ubiquitous in nature and many are involved in the biosynthesis of pharmacologically important amines. For example, the physiological products for the reactions catalysed by glutamic acid decarboxylase (GAD), aromatic amino acid decarboxylases and histidine decarboxylase are γ-aminobutyric acid (GABA), dopamine and histamine, respectively.

In view of their potential as targets for chemotherapeutic agents, suicide inhibitors have been designed and prepared for almost all of these enzymes.[2] In several cases the mechanistic



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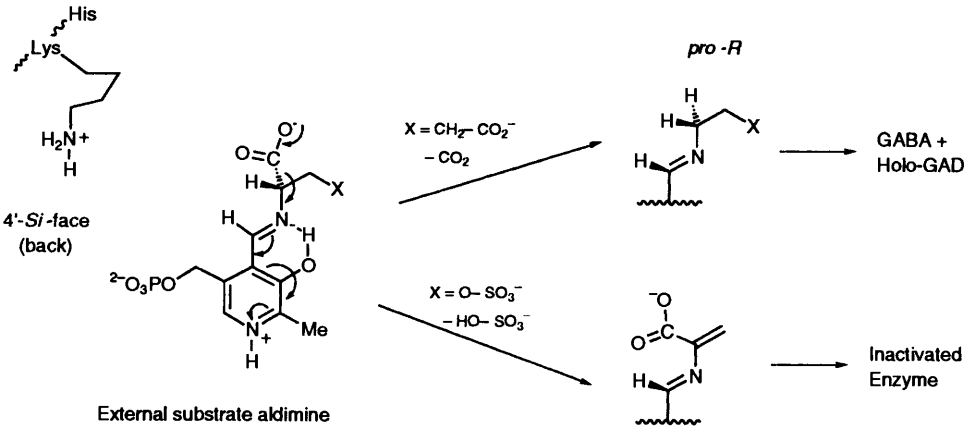
and stereochemical features of the suicide inactivation processes have been difficult to rationalise within the context of the known properties of pyridoxal dependent systems. In certain cases bonds connected to C^{α} were *apparently* cleaved on the wrong and unexpected 4'-*Re*-face of the coenzyme, for example, for acetylenic GABA [4-aminohex-5-ynoic acid **1**][3,4] and (2*S*)-serine *O*-sulfate **2**,[5] Scheme 1 (for full details see ref. 2). Recent studies on the inactivation of *E. coli* GAD by (2*S*)-serine *O*-sulfate[6] have confirmed the stereochemical implications of the earlier observations and, indeed, it is the α-proton rather than the carboxy group that is cleaved[7] (see following article in this issue).

In order to define the conformations of substrates and inhibitors and, hence, the positions of the distal binding groups at the active-site of GAD from *E. coli*, three stereochemical investigations were considered.

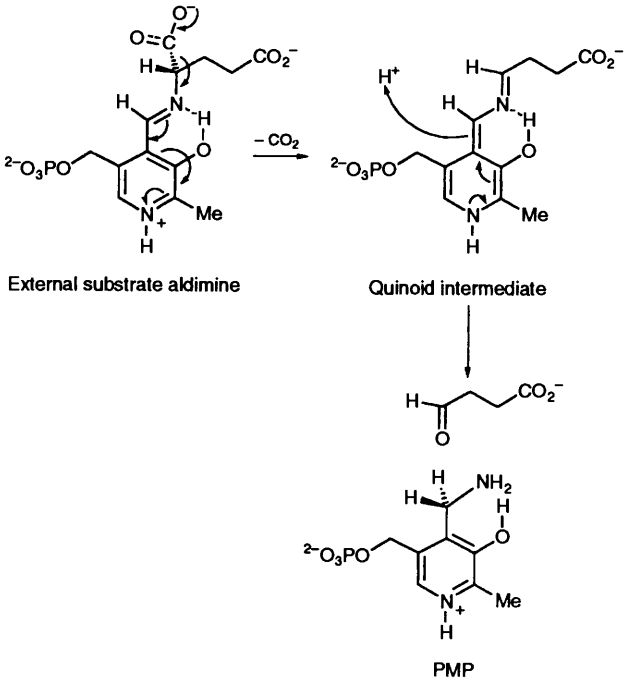
In the first, experiments were devised to determine the facial selectivity for proton transfer to the C-4' position of the coenzyme during an abortive decarboxylation–transamination reaction catalysed by the enzyme (Scheme 2). The outcome was expected to reveal which face of the coenzyme was exposed to the proton donor and hence allow a comparison with the results obtained for methionine decarboxylase and for transaminase enzymes.[8]

In the second, experiments were designed to determine the stereochemical course and the fidelity of decarboxylation and reprotonation events at C^{α} of the quinonoid intermediate derived from a 'loose fit' substrate, (2*S*)-aspartic acid. If the 'loose-fit' substrate showed the same retentive stereochemical course as for the natural substrate and, if the reaction showed high stereochemical fidelity, then two explanations would be likely. One rationalisation would be that the same conjugate acid-proton donor operates at C^{α} and C-4'. Alternatively, a similar outcome would be expected if two acids acted, one at C^{α} and one at C-4', and if the acids resided on the same face of the coenzyme, as was observed for methionine decarboxylase.[9]

In the third approach, it was intended to assess the ability of *E. coli* apoglutamic acid decarboxylase to reactivate itself in the presence of $N^{4'}$-(2''-phosphoethyl)pyridoxamine 5'-phosphate **3**. A parallel reaction had been reported for the mammalian brain enzyme[10,11] and, if the *E. coli* enzyme displayed similar properties, experiments could be designed to determine the stereochemical preference for a proton abstraction step in the regeneration of the holoenzyme from apoenzyme and $N^{4'}$-(2''-phosphoethyl)pyridoxamine 5'-phosphate **3**. Ultimately, a knowledge of the stereochemical preference for proton abstraction would allow the relative positions of the distal binding groups of several different enzyme-bound species including substrates, inhibitors and activators to be compared.



Scheme 1



Scheme 2

Table 1 Determination of the absolute configuration at C-4′ of the 4′-tritiated pyridoxamine derived from the abortive transamination reaction mediated by glutamic acid

	pH 6.0 (t = 16 h)	
	Enzymic	Nonenzymic[a]
dpm nmol^{-1} in pyridoxamine	50 000 (100%)	50 120 (100%)
dpm nmol^{-1} recovered in pyridoxal	47 880 (95%)	26 062 (52%)
dpm present in water	2 520 (5%)	24 058 (48%)

[a] Chemical method.

Results and Discussion

The stereospecificity of the protonation of the quinonoid intermediate at C-4′ during abortive decarboxylation–transamination was determined using the natural substrate, (2*S*)-glutamic acid. An earlier study had employed (2*RS*)-2-methylglutamic acid to perform a similar stereochemical correlation and the results had indicated that 4′-H_S of pyridoxamine 5′-phosphate was introduced during the transamination.[12] Unfortunately, it could not be assumed that the same stereochemical course would have been followed in the much less frequent transamination which occurs with the physiological substrate.[12] Indeed, for *E.* coli glutamate decarboxylase there was some question as to whether the same or different proton-donating groups acted upon the quinonoid intermediates derived from (2*S*)-glutamic acid and (2*RS*)-2-methylglutamic acid, during the formation of the respective amine products.[13] In view of these complications [the analogues' large size, its very low V_{max} value, 10^3-fold lower than (2*S*)-glutamic acid[12] and its racemic nature] we were compelled to use the natural substrate, (2*S*)-glutamic acid.

Accordingly, [4′-^{3}H]-PLP was prepared as described previously[14] and was incubated with *E. coli* GAD at pH 6.0 in the presence of (2*S*)-glutamic acid. Separation of the tritiated PMP transamination product from the incubation mixture, followed by treatment with alkaline phosphatase gave the 4′-tritiated pyridoxamine. After purification, the absolute configuration at C-4′ in the sample was determined using apoaspartate aminotransferase[14] which is known to exchange the 4′-*pro*-*S* hydrogen of pyridoxamine with solvent.[15] None of the tritium was exchanged into the solvent in the sample derived from the decarboxylation incubation whereas 50% of the tritium in a racemic synthetic sample was exchanged (see Table 1). This result indicates that a proton is transferred to the 4′-*Si*-face of the coenzyme during the abortive transamination (Scheme 2) in accord with the results obtained for methionine decarboxylase.[14] Interestingly, this result also indicates that there is no change in stereochemical preference for the protonation at C-4′ of the coenzyme on increasing the steric bulk at C$^\alpha$ of the substrate from a H-atom to a methyl group.[12]

In order to determine the stereochemical course of the protonation at C$^\alpha$ in the quinonoid intermediate derived from a 'loose-fit' substrate, (2*S*)-[2-^{2}H]aspartic acid and unlabelled aspartic acid were incubated with *E. coli* GAD in protium oxide and in deuterium oxide, respectively. When the decarboxylations were complete the labelled β-alanine products were purified by ion-exchange chromatography and were each converted into their *N*-(1*S*,4*R*)-camphanamide derivatives. Analysis of the NMR spectra (Fig. 1) and mass spectra of the derivatives and comparison of the NMR spectra with those obtained for synthetic C-3 chirally deuteriated samples[16] indicated that the decarboxylation occurred stereospecifically and with retention of configuration at C$^\alpha$ of the substrate. The high chiral integrity of the decarboxylation products is in keeping with the notion that either a single acid or two acids operate on the 4′-*Si*-face of the coenzyme. These results, *i.e.* high chiral integrity and retention of configuration, are

analogous to those obtained for methionine decarboxylase (which has been probed with a wide range of substrates and at extreme pH's)[9,14] and suggest that the enzymes share common active-site structural features. Unfortunately, *E. coli* GAD is not stable above pH 6.0[17,18] and, therefore, it has not been possible to test the stereospecificity for protonation at C^{α} at high pH as it was in the case of methionine decarboxylase.[9,14]

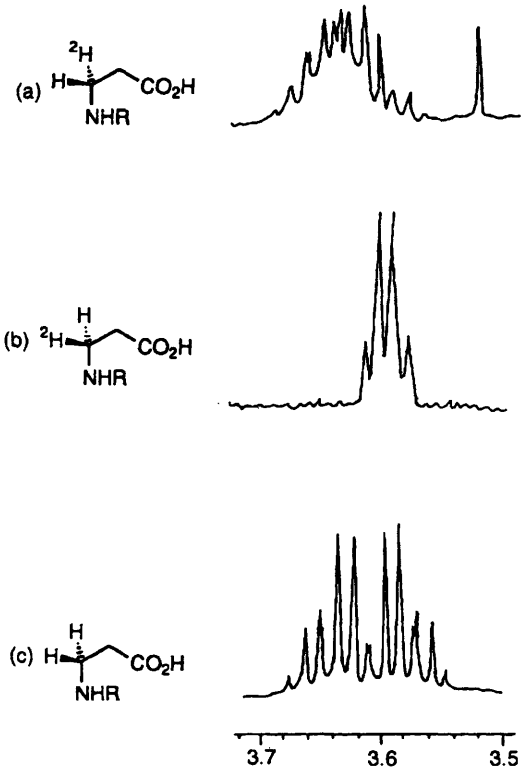
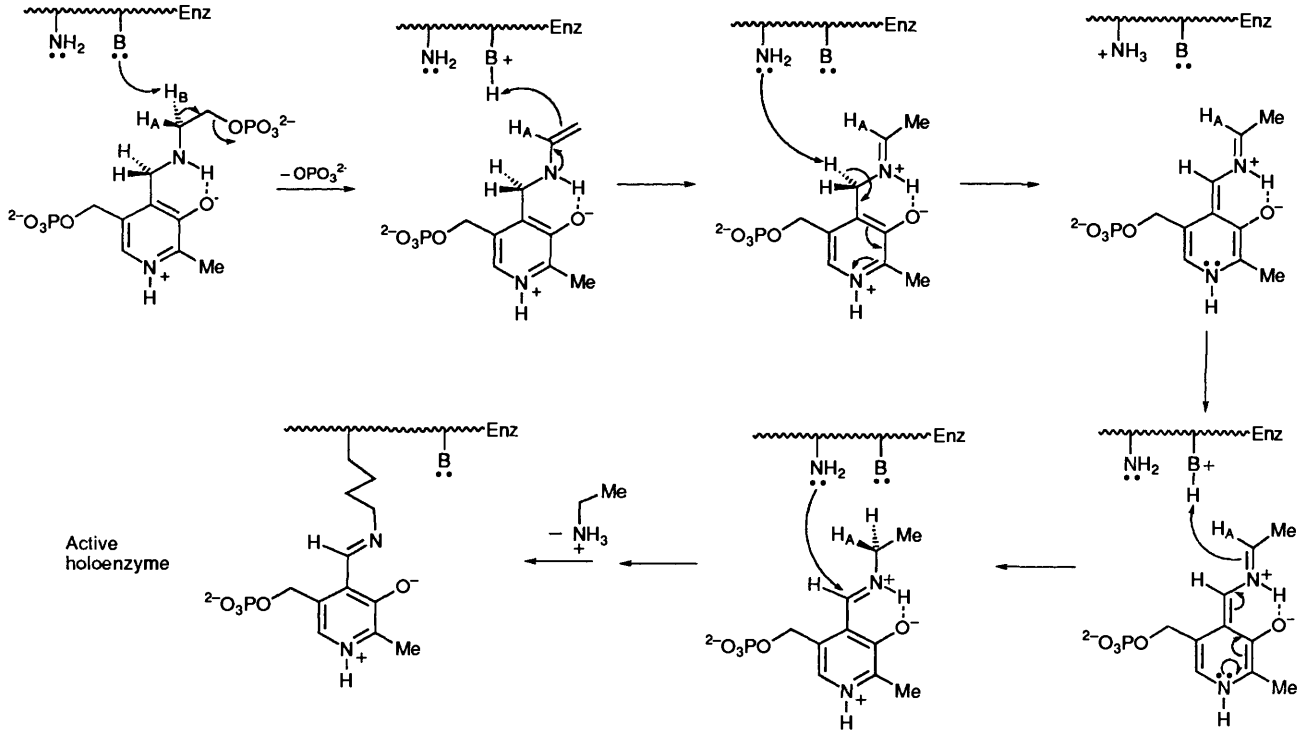


Fig. 1 ^{1}H NMR spectra of the C-3 protons of (1*S*,4*R*)-*N*-campanoyl-β-alanine isolated from an incubation of (a) (2*S*)-aspartic acid in ^2H_2O; (b) (2*S*)-[$^2H_1$]-aspartic acid in H_2O; (c) (2*S*)-aspartic acid in H_2O, with *E. coli* GAD

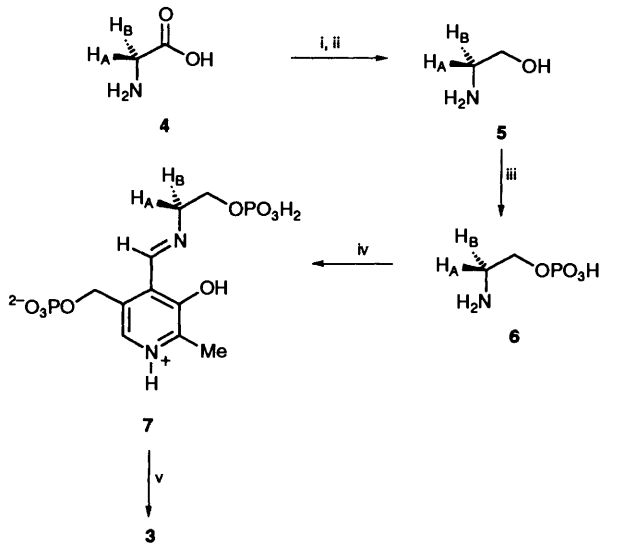
Reactivation of GAD Apoenzyme.—The regeneration of active holoenzyme from apoenzyme and $N^{4'}$-(2″-phosphoethyl)-pyridoxamine 5′-phosphate **3** had not been reported for *E. coli* GAD. Nevertheless, it had been demonstrated that the compound could reactivate the porcine brain enzyme[10] and a mechanism involving the elimination of phosphoric acid from the phosphoethyl moiety (to give PLP and ethylamine) had been proposed[11] (Scheme 3). Hence, $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **3** was prepared, through borohydride reduction of the aldimine formed from PLP and 2-aminoethyl phosphate, and was incubated with freshly prepared *E. coli* glutamic acid decarboxylase apoenzyme, see below. Aliquots of the enzyme solution were removed over a period of several hours and were assayed for activity using (2*S*)-[1-^{14}C]glutamic acid in a standard assay[19] at pH 4.6 (see Experimental section). Active enzyme was slowly generated and in this respect the bacterial enzyme was similar to the mammalian enzyme.[10,11]

In order to determine the stereochemical course of the abstraction step in the elimination, it was first necessary to develop a synthetic route to $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate which would allow the stereospecific incorporation of deuterium at C-1 of the phosphoethyl group.

Accordingly, glycine was esterified using methanol in the presence of thionyl chloride (Scheme 4). Reduction of the methyl ester with lithium aluminium hydride in THF gave ethanolamine **5** in 52% yield from glycine. Selective phosphorylation of the alcohol using phosphoric acid under dehydrating conditions (high temperature and reduced pressure), followed by purification of the phosphoester using ion exchange chromatography (Dowex 1 formate) gave ethanolamine phosphate **6** in 60% yield. Condensation of the phosphate **6** with the disodium salt of pyridoxal 5′-phosphate in methanol in the dark, followed by *in situ* reduction of the resulting aldimine using an excess of sodium borohydride gave the desired secondary amine bis-phosphate **7** as a hydrochloride salt. Purification using ion exchange chromatography (DEAE



Scheme 3



Scheme 4 i, MeOH, $SOCl_2$, reflux, 10 min, 94%; ii, $LiAlH_4$, tetrahydrofuran, reflux, 2 h, 55% (after distillation); iii, H_3PO_4, 100 °C, 10 mmHg, 3 h, then Amberlite 1R 120-H chromatography, 60%; iv, PLP, MeOH, reflux in the dark; v, $NaBH_4$, O °C, 10 min, then Sephadex A-25 cation exchange chromatography, 30% over iv and v

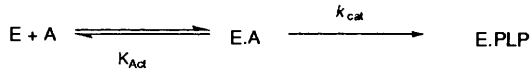
Sephadex A-25-100) gave pure $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **3** in 30% yield over the two steps from the phosphate **6**. The ^1H NMR spectrum showed a new signal at 7.6 ppm for the 6-H proton. The chemical shift for this signal is very sensitive to the functionality at C-4′ in the pyridoxamine nucleus and the absence of other signals in this region of the spectrum, together with other data, see Experimental section, indicated that the product **3** was pure.

In order to synthesise the chirally deuteriated isotopomers **3**(H_A = ^2H, H_B = H; and H_A = H, H_B = ^2H), (2*S*)- and (2*R*)-[2-$^2H_1$]glycine **4**(H_A = ^2H, H_B = H) and **4**(H_A = H, H_B = ^2H) were prepared from glycine and [2-$^2H_2$]glycine, respectively, using commercial preparations of glutamic pyruvic transaminase (GTP, E.C. 2.6.1.2) and the appropriately labelled water. This enzyme is known to catalyse the reversible exchange of the 2-*pro* (*R*) hydrogen of glycine with solvent hydrogen.[20] The chirally deuteriated samples of glycine and [2-$^2H_2$]glycine were then each converted into the corresponding $N^{4'}$-(2″-phosphorethyl)pyridoxamine 5′-phosphates **3**(H_A = ^2H, H_B = H; H_A = H, H_B = ^2H, and; H_A = H_B = ^2H) as outlined in Scheme 4. The chirally deuteriated and dideuteriated samples and their intermediates showed the expected mass and NMR spectral properties.

In order to prepare apoglutamic acid decarboxylase suitable for kinetic studies of the reactivation, the holoenzyme was subjected to abortive transamination using 100 mmol dm^{-3} (2*RS*)-2-methylglutamic acid according to the method of Yang and Metzler.[21] This treatment converts the coenzyme into PMP which cannot form an internal aldimine linkage to the enzyme's active site Lys residue and is, therefore, easy to separate from the enzyme. As noted above (2*RS*)-2-methylglutamic acid converts the holoenzyme into apoenzyme *via* transamination much more frequently than (2*S*)-glutamic acid.[12] The PMP that was formed during the reaction was removed by dialysing the enzyme–inactivator mixture for 12 h at 4 °C in 1 mol dm^{-3} aqueous glycerol containing 0.1 mmol dm^{-3} dithiothrietol. Under these conditions the apoenzyme loses minimal reconstitutable holoenzyme activity. The apoenzyme produced in this manner contains a residual holoenzyme activity of only 2% of the original activity while >80% of the original holoenzyme activity can be obtained through the reconstitution with PLP.

Each of the four $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphates **3**(H_A = H_B = H; H_A = ^2H, H_B = H; H_A = H, H_B = ^2H, and; H_A = H_B = ^2H) were incubated, in duplicate, at a range of four concentrations (10, 20, 100 and 500 μmol dm^{-3}) with 2.6 μmol dm^{-3} of apoGAD. After exactly 3 h at 30 °C, aliquots of each of the enzyme solutions were removed and were assayed for activity using (2*S*)-[1-^{14}C]glutamic acid at pH 4.6. (Initial trial experiments were performed to estimate the best time to perform accurate activity determinations).

The activation was modelled using Scheme 5 where E_{Apo}(Tot)



Scheme 5

is the total starting amount of inactive enzyme defined according to Equation (1).

$$E_{Apo}(Tot) = E_{Apo} + E_{Apo}.A \quad (1)$$

Scheme 5 will show saturation kinetics [eqn. (2)] but, the

$$v = [A][E_{Apo}(Tot)]k_{cat}/([A] + K_{Act}) \quad (2)$$

initial rate, *v*, cannot be determined directly. This is because, unusually, the starting system is inactive and at very small times there is no activity to measure.

(Note that the corrected, not the *actual*, activity at $t = 0$ is zero and that the residual activity correction is simply implemented by subtracting the activity measured in controls containing no added activator, compound **3**.) Thus, the most accurate experimental activity determinations must be performed after significant reactivation has occurred (*i.e.* after several hours) but before significant non-specific irreversible inactivation of the apoenzyme occurs. The activation reaction is a pseudo-first order process when activator concentrations are much higher than the concentration of apoenzyme, essentially the conditions described here.

The apparent rate constants for activation were determined from the negative slopes of plots of ln {[E_{Apo}]-[E.PLP]} *vs.* time where the concentration of active enzyme, [E.PLP], was determined directly by measuring decarboxylase activity in the standard assay, see below. From the increase in gradient (-k^{App}) with increasing activator (compound **3**) concentration, [A], the values for k_{cat} and K_{Act} were obtained by fitting to eqn. (3) (see Table 2).

$$k^{App} = [A]k_{cat}/([A] + K_{Act}) \quad (3)$$

The kinetic data for the reactivation reaction with the unlabelled compound **3**(H_A = H_B = H) gave k_{cat} and K_{Act} values of 1.6×10^{-5} s^{-1} and 50 μmol dm^{-3}, respectively. The corresponding data for the dideuteriated compound **3**(H_A = H_B = ^2H) was $k_{cat} = 0.9 \times 10^{-5}$ 3^{-1}; and K_{Act} = 45 μmol dm^{-3}. This shows that the active enzyme is generated more slowly than for the unlabelled compound. Thus, there is a primary deuterium isotope effect of ~1.7 for the removal of a C-1 hydrogen from the activator which is expressed approximately equally on k_{cat} and k_{cat}/K_{Act}. From Table 2 it is also evident that the two C-1 chirally deuteriated isotopomers possess different kinetic properties. The (1*S*)-[1-$^2H_1$]-$N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **3**(H_A = ^2H, H_B = H) mediated reactivation *E. coli* apoGAD gives reaction rates identical, within experimental error, with the values obtained for the unlabelled compound. Conversely, the (1*R*)-antipode displays rates very similar to those obtained for the dideuteriated compound (see Table 2). Therefore, the enzyme removes

Table 2 Kinetic parameters obtained for the reactivation of apoGAD by isotopomers of $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **3**

Activator	K_m μmol dm^{-3}	k_{cat} s^{-1}
Unlabelled **3**	52 ± 5	1.58×10^{-5}
Dideuteriated **3** ($H_A = H_B = {}^2H$)	45 ± 5	0.91×10^{-5}
(*R*)-Monodeuteriated **3** ($H_A = H$, $H_B = {}^2H$)	48 ± 5	0.89×10^{-5}
(*S*)-Monodeuteriated **3** ($H_A = {}^2H$, $H_B = H$)	47 ± 5	1.64×10^{-5}

Errors for k_{cat} are ≤8% of stated values.

the 1-*pro-R* hydrogen from the phosphoethyl group of $N^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **3** during the reactivation process. Notably, this hydrogen atom is expected to occupy the spatially equivalent position to that occupied by the proton that is introduced into the quinonoid intermediate in the formation of γ-aminobutyric acid from the natural substrate, (2*S*)-glutamic acid. Note that the 3-*pro-R* hydrogen of the (2*S*)-aspartic acid decarboxylation product, β-alanine, is also expected to occupy this position, on the 4′-*Si*-face of the coenzyme (at C$^{\alpha}$), if all of the distal anionic binding groups occupy similar positions on the 3-OH side of the pyridine heterocycle.

Location and Identity of Conjugate Acids.—It was already established that borohydride reduction of the *E. coli* GAD holoenzyme aldimine complex occurred *via* hydride transfer to C-4′ to give a pyridoxyllysine peptide.[22] Thus, it is highly probable that the proton donor for C-4′ of the quinonoid intermediate during abortive decarboxylation–transamination is the ε-ammonium group of the Lys residue. This idea is supported also by the fact that the ε-ammonium group of the active site Lys residue of aspartate aminotransferase is known to shuttle protons between the C$^{\alpha}$ and C-4′ positions of intermediate quinonoids.[8] However, it is far from clear that the ε-ammonium group of the Lys residue can also protonate the quinonoid intermediate at C$^{\alpha}$ in decarboxylase enzymes. Indeed, several lines of evidence indicate that the conjugate acid for the protonation at C$^{\alpha}$ during the normal course of decarboxylation is the imidazolium side chain of a His residue.

First, a His residue has been shown to fulfil an identical function in eukarotic and prokaryotic methionine decarboxylase.[9,14,23] Second, chemical modification studies by Mishin and Sukhareva[24] have identified a catalytically essential His residue which is not involved in binding to the substrate in the Michaelis complex. Third, the enzyme shows large solvent deuterium isotope effects of approximately 5.0 and 2.6 for ^{D}V and $^{D}(V/K)$ respectively[18,25] yet the amine product isolated from incubations conducted in partially labelled solvent contains the same isotope content as the solvent.[26] Since there is no evidence at all to indicate that protonation at C$^{\alpha}$ occurs at equilibrium (*i.e.* is followed by a very slow step) the most reasonable explanation is that a monoprotic conjugate acid operates at C$^{\alpha}$.

In conclusion, for *E. coli* glutamate decarboxylase it appears that the lysyl ε-ammonium group and histidyl imidazolium groups are responsible for the specific protonation of the quinonoid intermediate at C-4′ and C$^{\alpha}$, respectively. Furthermore, on the basis of the results obtained for aspartic acid, which show that decarboxylation occurs with retention of configuration and with high fidelity, it appears that these conjugate acids are disposed on the 4′-*Si*-face of the coenzyme. Thus, the distal anionic binding groups of substrates and substrate analogues must occupy similar positions on the 3′-OH side of the coenzyme. This interpretation suggests that the unexpected '4′-*Re*-face' reactions observed for GAD with the suicide substrates (*S*)-acetylenic GABA and (2*S*)-serine *O*-sulfate do not result from the availability of alternative binding modes. However, the reactions may be promoted by the enhanced acidity of the flanking multiply bonded moieties and, in actual fact, the enzymes may not provide a base on the 4′-*Re*-face of the coenzyme at all. The following article addresses these issues.

Experimental

Elemental analysis were carried out at the microanalytical laboratory, University College, London. Specific rotations were determined on an optical Activity AA-100 polarimeter using 5 cm pathlength cell at 21 °C, and are given in units of 10^{-1} deg cm^2 g^{-1}. All ^{1}H NMR spectra were recorded on either a JEOL JNM-GX270 (270 MHz), a Bruker AM360 (360 MHz) or a Varian VXR500 (500 MHz) spectrometer. Aqueous solutions were referenced using either the H^{2}HO signal (at δ 4.61), the signal for 1,4-dioxane (at δ 3.66), or the sodium salt of 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionic acid [TMS(Na)] signal (at δ 0.0). *J* Values are given in Hz. {^{1}H} ^{13}C NMR spectra were recorded at 67.9 MHz, and chloroform (δ_C 77.20), 1,4-dioxane (δ_C 67.40), or methanol (δ_C 47.00) were used as chemical-shift references. Mass spectra and accurate mass measurements were recorded on a Kratos MS 30 or a VG 70 250 SE spectrometer. Major fragments are given as percentages of the base peak intensity (100%). Fast-atom bombardment (FAB) spectra were recorded using glycerol as matrix.

Amino acid substrates, PLP, PMP, buffers, salts, deuterium oxide, alkaline phosphatase, glutamic acid decarboxylase and acylase 1 were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Aspartate aminotransferase (glutamate-oxaloacetate transaminase, GOT) and glutamate-pyruvate transaminase (GTP) were obtained from Boehringer Mannheim (Lewes, Sussex, UK). Amberlite IR 120 (H$^+$), DEAE Sephadex A25 120 and Dowex 1x8 (OH) ion exchange resins were obtained from British Drug Houses (Poole, Dorset, UK) and [1-^{14}C]-L-amino acid substrates, sodium [^{3}H]borohydride and tritiated water were obtained from Amersham International (Amersham, Bucks, UK). Water-miscible scintillant (ES-199) was obtained from Canberra Packard (Pangbourne, Berks, UK). All other chemicals were of analytical grade or were recrystallised or redistilled before use.

Stereochemical Course of Transamination.—(4*RS*)-[4′-$^3H_1$]-PMP and [4′-^{3}H]-PLP were prepared by a modification of the methods of Voet *et al.*[27] and Metzler *et al.*[28] as described by Stevenson *et al.*[14]

(i) *Formation of tritiated PMP.*[14] *E. coli* glutamic acid decarboxylase (2–4 units, freed of unbound coenzyme[21]) was incubated with (2*S*)-glutamic acid (25 mg, 0.17 mmol) and [4′-^{3}H]-PLP (98 nmol, 10^8 dpm) in 100 mmol dm^{-3} potassium succinate or phosphate buffer (5 cm^3) at pH 6.0 for 14 h at 37 °C. The reactions were followed by TLC analysis on cellulose eluting with propan-2-ol–aqueous NH_3(*d* 0.88)–water (26:6:5). When no unchanged glutamate remained the solution was diluted to 10 cm^3 with water and unlabelled PMP (1.3 mg, 5.2 μmol) was added to it. The pH was adjusted to 11.0 with 500

mmol dm^{-3} aqueous sodium hydroxide and the tritiated PMP was isolated exactly as described previously above.

(ii) *Conversion to pyridoxamine.* The sample of PMP was incubated with alkaline phosphatase (10 units) in 100 mmol dm^{-3} ammonia solution adjusted to pH 10 with acetic acid. After 20 h, the phosphate ester hydrolysis was complete as judged by TLC on silica. The samples of tritiated pyridoxamine were purified on Dowex 1x8 pre-equilibrated with 100 mmol dm^{-3} ammonia solution adjusted to pH 10.6. The samples were washed with equilibration buffer and eluted from the column in the same buffer at pH 8.0. The fractions containing pyridoxamine were combined. The radioactivity of the sample was determined by liquid scintillation counting.

(iii) *Incubation with ApoAAT.* In order to determine the stereochemical integrity at C-4′ a small portion of the sample of pyridoxamine (~ 50 000 dpm) was incubated with apoaspartate aminotransferase (1 mg) prepared according to the method of Yang and Metzler using the reaction conditions of Dunathan[29] and the extent of tritium release into the water was determined. The tritium content at C-4′ of the pyridoxal produced was also determined (see Table 1). A control containing synthetic (4*RS*)-[4′-$^3H_1$]-PMP was also incubated with apoaspartate aminotransferase.

(2S)-[2-$^2H_1$]*Aspartic Acid.*—This compound was prepared by solvent hydrogen exchange catalysed by glutamate-oxaloacetic transaminase (GOT) according to the method of Rose *et al.*[7] using (2*S*)-aspartic acid (3.0 g, 22.5 mmol) in 88% yield; m.p. > 300 °C (decomp.) (Found: C, 27.95; H, 5.3; N, 8.55. $C_4H_9ClNO_4$ requires C, 28.15; H, 5.3; N, 8.2%); $[\alpha]_D$ +23.5 (*c* 0.95 in 6 mol dm^{-3} HCl) {lit.,[30] $[\alpha]_D$ +24.6 (6 mol dm^{-3} HCl) for unlabelled aspartic acid}; δ_H(270 MHz; ^2H_2O) 2.5 (2 H, d, *J* 2.9, 3-CH_2) and 4.15 (1/40 H, t, 2-CH); *m/z* (FAB-glycerol–acetic acid matrix) 135 ($[M + Cl]^+$, 100) and 89 (20, $C_3H_7NO_2^+$).

(1′S,4′R)-N-*Camphanoyl*-β-*alanine.*—β-Alanine (100 mg, 1.12 mmol) was dissolved in 1 mol dm^{-3} aqueous NaOH (2.5 cm^3) and (1*S*,4*R*)-camphanoyl chloride (150 mg, 0.81 mmol) in toluene (2.5 cm^3) was added to the solution. The mixture was stirred vigorously overnight after which it was diluted with water (5 cm^3) and the organic phase discarded. The aqueous phase was adjusted to pH 2 with 6 mol dm^{-3} HCl and extracted with dichloromethane (3 × 5 cm^3). The organic layers were combined, dried ($MgSO_4$) and concentrated under reduced pressure to yield a white solid which was recrystallised from dichloromethane–light petroleum, m.p. 128–130 °C (lit.,[16] 128–130 °C); δ_H(270 MHz; C^2HCl_3) 0.92 (3 H, s, 4′-CH_3), 1.07 (6 H, s, 7′- 2 × CH_3), 1.6–2.53 (4 H, m, 5′ and 6′-CH_2), 2.64 (2 H, t, *J* 6.1, 2-CH_2), 3.60 (2 H, m, 3-CH_2), 5.91 (1 H, br s, CO_2H) and 7.00 (1 H, br t, NH); *m/z* (EI) 269 (M^+) and 223 ($[M - HCO_2H]^+$). All spectroscopic data were identical with those described earlier.[16]

Determination of the Stereochemistry of Decarboxylation of Aspartic Acid by Glutamate Decarboxylase (GAD).—(a) *Incubation of* (2S)-*aspartic acid in* ^2H_2O *with* E. coli GAD. (2*S*)-Aspartic acid (25 mg, 180 nmol), GAD (5 mg, 100 units) and pyridoxal phosphate (0.02 mg, 100 nmol) were dissolved in $[^2H_2]$water (5 cm^3), and the solution was lyophilized. The residue was dissolved in pyridinium chloride buffer (200 mmol dm^{-3}, pH 4.0) in $[^2H_2]$water (5 cm^3) and the solution was incubated in the dark at 37 °C for 16 h. The pH was adjusted to 10 using 2 mol dm^{-3} aqueous KOH, and the protein was precipitated by the addition of ethanol, and then removed by centrifugation. The supernatant solution was concentrated under reduced pressure to give a white solid which was dissolved in water and then applied to a Dowex 1x8-200 (formate) ion exchange column. The column was eluted with a stepwise gradient of formic acid, consisting of 5 cm^3 portions of 0, 0.2, 0.5 and 1 mol dm^{-3} formic acid. The fractions containing β-alanine [R_F 0.5; eluting solvent, isopropyl alcohol–aq. ammonia (0.88 *d* cm^3)–water (26:6:5)] were pooled and reduced in volume under reduced pressure to give the decarboxylation product as a white solid. The deuteriated β-alanine was converted into its camphanoyl derivative, as described above. The derivative showed the following spectral data: *m/z* (EI) 270 (M^+); δ_H(270 MHz; C^2HCl_3) (β-alanine portion only) 2.64 (2 H, t, *J* 6.1, 2-CH_2) and 3.63 (1 H, m, 3-CH_S). Comparison of the ^1H NMR spectral data (Fig. 1a), with reported chemical-shift values[16] indicated that the decarboxylation occurred with retention of configuration.

(b) *Incubation of* (2S)-$[^2H_1]$*aspartic acid in* H_2O *with* E. coli GAD. (2S)-$[^2H_1]$aspartic acid (25 mg, 180 nmol), GAD (5 mg, 100 units) and PLP (0.02 mg, 100 nmol) were dissolved in pyridinium chloride buffer (200 mmol dm^{-3}, pH 4.0) made up in water (5 cm^3). The solution was incubated in the dark for 16 h at 37 °C. The crude β-alanine was purified, derivatised as its camphanic acid amide and analysed as for the (2*S*)-aspartic acid incubation above. The derivative showed the following spectral data: *m/z* (EI) 270 (M^+); δ_H(270 MHz; C^2HCl_3) (β-alanine portion only) 2.64 (2 H, t, *J* 6.1, 2-CH_2) and 3.57 (1 H, m, 3-CH_R). Thus, the β-carbon is of the *S*-configuration verifying that the decarboxylation occurred with retention of configuration (Fig. 1b).

(2S)-[2-$^2H_1$]*Glycine* **4**(A = ^2H, B = H).—Following the method of Gani *et al.*[31] [2-$^2H_2$]glycine (1 g, 13 mmol), dipotassium hydrogen phosphate (70 mg, 6.4 mmol), pyridoxal 5′-phosphate (3 mg, 15 mmol) and GTP (1 mg, 1000 units) were dissolved in water, the pH of the solution being adjusted to 7.1 with 35% HCl before it was incubated in the dark at 37 °C for 9 days. The enzyme was denatured by boiling for 2 min and removed by centrifugation. The supernatant solution was lyophilized to yield a white crystalline solid (900 mg, 99%). The monodeuteriated glycine showed the expected ^1H NMR spectral parameters and a small sample was fully characterised as the (1*S*,4*R*)-*N*-camphanoyl derivative.[31]

(2R)-[2-$^2H_1$]*Glycine* **4**(A = H, B = ^2H).—Glycine (1 g, 13 mmol) was dissolved in $[^2H_2]$water (5 cm^3) and the solution was lyophilised. The residue was redissolved in deuterium oxide (5 cm^3) and dipotassium deuterium phosphate (70 mg, 6.4 mmol), pyridoxal 5′-phosphate (3 mg, 15 mmol) and GTP (1 mg, 1000 units) were added to the solution, the pH of which was then adjusted to 7.1 with 35% ^2HCl. The solution was then incubated in the dark at 37 °C for 9 days. The monodeuteriated glycine was isolated as described above and showed the expected ^1H NMR spectral parameters. A small sample was characterised fully as its (1*S*,4*R*)-*N*-camphanoyl derivative.[31]

Glycine Methyl Ester Hydrochloride.—Thionyl chloride (1 g, 14 mmol) was added dropwise to an ice-cold suspension of glycine (1 g, 13 mmol) in dry methanol (20 cm^3). The mixture was allowed to warm to room temperature, and then refluxed for 30 min to give a clear solution. The solution was cooled to 0 °C and diluted with dry ether until crystals appeared. The crystals were filtered off and washed thoroughly with ice-cold dry ether to give glycine methyl ester hydrochloride (1.1 g, 88%), m.p. 171–173 °C (lit.,[32] 175 °C); ν_{max}(Nujol)/cm^{-1} 3350 (NH) and 1745 (C=O); δ_H(270 MHz; ^2H_2O) 3.83 (3 H, s, OCH_3) and 3.92 (2 H, s, 2-CH_2); *m/z* (EI) 89 ($[M - HCl]^+$, 6%) and 30 (100, $[M - HCl - COOCH_3]^+$).

(2S)-[2-$^2H_1$]*Glycine methyl ester hydrochloride.* This compound was prepared in a manner identical with that used for the unlabelled compound, starting from (2*S*)-[2-$^2H_1$]glycine; m.p. 171–173 °C (lit.,[32] 175 °C, for unlabelled product); ν_{max}(Nu-

jol)/cm^{-1} 3350 (NH) and 1745 (C=O); δ_H(270 MHz; ^2H_2O) 3.82 (3 H, s, OCH_3) and 3.91 (1 H, s, 2-CH); δ_H(67.9 MHz; ^2H_2O) 42 (2-CH), 56 (OCH_3) and 170 (CO_2H); *m/z* (EI) 90 ([M − HCl]$^+$, 6%) and 31 (100, [M − HCl − CO_2CH_3]$^+$).

(2R)-[2-$^2H_1$]*Glycine methyl ester hydrochloride*. This compound was prepared in a manner identical with that used for the unlabelled compound, starting from (2*R*)-[2-$^2H_1$]glycine; m.p. 174–176 °C (lit.,[32] 175 °C, for unlabelled product); ν_{max}(Nujol)/cm^{-1} 3350 (NH) and 1745 (C=O); δ_H(270 MHz; ^2H_2O) 3.82 (3 H, s, OCH_3) and 3.91 (1 H, s, 2-CH); δ_H(67.9 MHz; ^2H_2O) 42 (2-CH), 56 (OCH_3) and 170 (CO_2H); *m/z* (EI) 90 ([M − HCl]$^+$, 6%) and 31 (100, [M − HCl − CO_2CH_3]$^+$).

[2-$^2H_2$]*Glycine methyl ester hydrochloride*. This compound was prepared in a manner identical with that used for the unlabelled compound, starting from [2-$^2H_1$]glycine; m.p. 171–173 °C (lit.,[32] 175 °C, for unlabelled product); ν_{max}(Nujol)/cm^{-1} 3350 (NH) and 1745 (C=O); δ_H(270 MHz; ^2H_2O) 3.82 (3 H, s, OCH_3); δ_H(67.9 MHz; ^2H_2O) 42 (2-CH), 56 (OCH_3) and 170 (CO_2H); *m/z* (EI) 91 ([M − HCl]$^+$, 8%) and 32 (100, [M − HCl − CO_2CH_3]$^+$).

2-*Aminoethanol* **5**(A = B = H).—To dry methyl glycinate hydrochloride (2 g, 16 mmol) was added slowly with stirring a solution of lithium aluminium hydride (850 mg, 20 mmol) in dry tetrahydrofuran (20 cm^3), over 5 min according to the method of Gani *et al.*[31] The reaction mixture was refluxed for 2 h and then cooled. Saturated aqueous Na_2SO_4 was slowly added dropwise until the effervescence ceased. The solids were separated and then extracted with tetrahydrofuran (300 cm^3) in a Soxhlet extractor for 18 h. The pale yellow solution was evaporated to give a heavy viscous oil, which was Kugelrohr distilled, to yield 2-aminoethanol as an almost colourless oil (0.47 g, 44%); b.p. 66–75 °C/10 mmHg (lit.,[31] 66–75 °C/10 mmHg); ν_{max}(Nujol)/cm^{-1} 3400 (NH); δ_H(270 MHz; ^2H_2O) 2.85 (2 H, t, 2-CH_2) and 3.55 (2 H, t, *J* 6, 1-CH_2); *m/z* (EI) 61 (M$^+$, 9%), 43 (12, [M − H_2O]$^+$) and 30 (100, [M − CH_2OH]$^+$).

(2S)-[2-$^2H_1$]*Aminoethanol* **5**(A = ^{2}H, B = H). This compound was prepared in a manner identical with that used for the unlabelled compound, starting from (2*S*)-[$^2H_1$]methyl glycinate hydrochloride; the alcohol was obtained as a colourless oil after distillation (55% yield), b.p. 70–75 °C/10 mmHg; ν_{max}(Nujol)/cm^{-1} 3400 (NH); δ_H(270 MHz; ^2H_2O) 2.80 (1 H, t, *J* 6, 2-CH) and 3.55 (2 H, d, *J* 6, 1-CH_2); *m/z* (EI) 62 (M$^+$, 8%), 44 (11, [M − H_2O]$^+$) and 31 (100, [M − CH_2OH]$^+$).

(2R)-[2-$^2H_1$]*Aminoethanol* **5** (A = H, B = ^{2}H). This compound was prepared in a manner identical with that used for the unlabelled compound, starting from (2*R*)-[$^2H_1$]methyl glycinate hydrochloride; the alcohol was obtained as a colourless oil after distillation (57% yield), b.p. 64–67 °C/10 mmgHg; ν_{max}(Nujol)/cm^{-1} 3400 (NH); δ_H(270 MHz; ^2H_2O) 2.80 (1 H, t, *J* 6, 2-CH) and 3.55 (2 H, d, *J* 6, 1-CH_2); *m/z* (EI) 62 (M$^+$, 8%), 44 (11, [M − H_2O]$^+$) and 31 (100, [M − CH_2OH]$^+$).

[2-$^2H_2$]*Aminoethanol* **5**(A = B = ^{2}H). This C-2 dideuteriated alcohol was prepared in a manner identical with that used for the unlabelled material, starting from [$^2H_2$]methyl glycinate hydrochloride; the alcohol was obtained as a colourless oil after distillation (57% yield), b.p. 68–75 °C/10 mmHg; ν_{max}(Nujol)/cm^{-1} 3400 (NH); δ_H(270 MHz; ^2H_2O) 3.55 (2 H, s, *J* 6, 1-CH_2); *m/z* (EI) 63 ([M$^+$, 9%), 45 (10, [M − H_2O]$^+$) and 32 (100, [M − CH_2OH]$^+$).

2-*Aminoethanol O-Phosphate* **6**(A = B = H).—To a concentrated solution of phosphoric acid (1.96 g, 17 mmol) at 70 °C was added 2-aminoethanol (1 g, 16 mmol). The mixture was heated *in vacuo* at 100 °C/10 mmHg for 1 h, and then for a further 3 h at 150 °C/0.3 mmHg. A colourless solid was obtained on cooling and this was dissolved in water, and applied to an Amberlite IR 120 (H$^+$) ion exchange resin (25 × 2.5 cm). The column was eluted with water and the effluent was analysed by TLC on cellulose using ninhydrin to visualise the product. The fractions containing the phosphorylated compound [R_F 0.2; eluting solvent, isopropyl alcohol–aq. ammonia (*d* 0.88)–water (26:6:5)] were pooled and reduced in volume under reduced pressure to give 2-aminoethanol *O*-phosphate as a white solid (1.35 g, 60%), m.p. 242–244 °C (lit.,[33] 240–242 °C); ν_{max}(Nujol)/cm^{-1} 3300 (NH) and 1150 (P=O); δ_H(270 MHz; ^2H_2O) 3.05 (2 H, s, *J* 6, 2-CH_2) and 3.95 (2 H, q, *J* 6, 1-CH_2); *m/z* (FAB-glycerol–acetic acid matrix) 142 ([M + H]$^+$).

(2S)-[2-$^2H_1$]-2-*Aminoethanol O-phosphate* **6**(A = ^{2}H, B = H). This compound, prepared in a manner identical with that used for the unlabelled compound, starting from (2S)-[2-$^2H_1$]-2-aminoethanol, was obtained as white solid after purification (62% yield), m.p. 240–242 °C; ν_{max}(Nujol)/cm^{-1} 3300 (NH) and 1140 (P=O); δ_H(270 MHz; ^2H_2O) 3.05 (1 H, t, *J* 6, 2-CH) and 3.95 (2 H, q, *J* 6, 1-CH_2); *m/z* (FAB-glycerol–acetic acid matrix) 143 ([M + H]$^+$).

(2R)-[2-$^2H_1$]-2-*Aminoethanol O-phosphate* **6**(A = H, B = ^{2}H). This compound, prepared in a manner identical with that used for the unlabelled compound, starting from (2R)-[2-$^2H_1$]-2-aminoethanol, was obtained as white solid after purification (64% yield), m.p. 240–242 °C; ν_{max}(Nujol)/cm^{-1} 3300 (NH) and 1150 (P = O); δ_H(270 MHz; ^2H_2O) 3.05 (1 H, t, *J* 6 2-CH) and 3.95 (1 H, q, *J* 6, 1-CH_2); *m/z* (FAB-glycerol–acetic acid matrix) 143 ([M + H]$^+$).

[2-$^2H_2$]-2-*Aminoethanol O-phosphate* **6**(A = ^{2}H, B = ^{2}H). The C-2 dideuteriated phosphate, prepared in a manner identical with that used for the unlabelled compound, starting from [2-$^2H_2$]-2-aminoethanol, was obtained as white solid after purification (65% yield), m.p. 238–242 °C; ν_{max}(Nujol)/cm^{-1} 3300 (NH) and 1150 (P=O); δ_H(270 MHz; ^2H_2O) 3.95 (2 H, q, *J* 6, 1-CH_2); *m/z* (FAB-glycerol–acetic acid matrix) 144 ([M + H]$^+$).

N$^{4'}$-(2″-*Phosphoethyl*)*pyridoxamine* 5′-*Phosphate* **7**(A = B = H). To a dry methanolic solution of the dipotassium salt of PLP (650 mg, 2 mmol) was added in small portions the potassium salt of 2-aminoethanol *O*-phosphate (360 mg, 1.5 mmol). The mixture was refluxed in the dark for 2 h, cooled at 0 °C, and then treated with sodium borohydride (56 mg, 1.5 mmol), which was added with stirring. The resulting solution was allowed to warm to room temperature after which it was acidified to pH 3 (using 6 mol dm^{-3} HCl) and then reduced in volume under reduced pressure to give the crude product as a yellow solid. The crude material was dissolved in 50 mmol dm^{-3} $NaHCO_3$ (100 cm^3) and the pH of the solution was adjusted to 8.0 with Na_2CO_3; it was then applied to a DEAE-Sephadex A-25-120 ion exchange column (50 × 2.5 cm; pre-equilibrated with 50 mmol dm^{-3} aqueous $NaHCO_3$, pH 8.0). The sample was eluted with a linear gradient of 50–500 mmol dm^{-3} aqueous $NaHCO_3$ (pH 8.0). The required fractions [R_F 0.4; eluting solvent, isopropyl alcohol–aq. ammonia (*d* 0.88)–water (26:6:5)] were pooled and reduced in volume under reduced pressure to give a pale yellow solid (122 mg, 30%), m.p. 165–167 °C; *m/z* (FAB) (Found: [M + H]$^+$ 373.0577. $C_{10}H_{19}N_2O_9P_2$ requires 373.0566); ν_{max}(Nujol)/cm^{-1} 3600–3200 (OH), 1150 (P=O) and 1035 (P–O–C); λ_{max}/nm, (0.1 mmol dm^{-3} $NaOH_{aq}$) 325 (ε/dm^3 mol^{-1} cm^{-1} 10 000) and 252 (7000); δ_H(270 MHz; ^2H_2O) 2.38 (3 H, s, 2-CH_3), 3.23 (2 H, t, *J* 5, N-CH_2), 3.93 (2 H, t, *J* 5, O-CH_2), 4.36 (2 H, s, 4′-CH_2), 4.78 (2 H, d, $J_{5'-H,P}$ 11, 5′-CH_2) and 7.62 (1 H, s, 6-H); δ_C(270 MHz; ^2H_2O) 17.71 (2-CH_3), 47.01 (N-CH_2), 50.49 (OCH_2), 61.99 (5′-CH_2), 63.91 (4′-CH_2), 126.8, 133.27, 137.19, 147.52 and 165.25 (2,3,4,5 and 6-C).

(1″S)-[1″-$^2H_1$]-N$^{4'}$-(2″-*Phosphoethyl*)*pyridoxamine* 5′*phosphate* **7**(A = ^{2}H, B = H). This compound, prepared in a

manner identical with that used for the unlabelled compound, starting from (2*S*)-[2-$^2H_1$]-2-aminoethanol *O*-phosphate, was obtained as a pale yellow solid (28% yield), m.p. 160–162 °C; ν_{max}(Nujol)/cm^{-1} 3600–3200 (OH), 1150 (P=O) and 1035 (P–O–C); δ_H(270 MHz; ^2H_2O) 2.38 (3 H, s, 2-CH_3), 3.23 (1 H, t, *J* 5, N-CH), 3.93 (2 H, t, *J* 5, O-CH_2), 4.36 (2 H, s, 4′-CH_2), 4.78 (2 H, d, $J_{5'\text{-H,P}}$ 11, 5′-CH_2) and 7.62 (1 H, s, 6-H); *m*/*z* (FAB-glycerol–acetic acid matrix) 374 ([M + H]$^+$).

(1″R)-[1″-$^2H_1$]-N$^{4'}$-(2″-*Phosphoethyl*)*pyridoxamine* 5′-*phosphate* **7**(A = H, B = ^{2}H). This compound, prepared in a manner identical with that used for the unlabelled compound, starting from (2*R*)-[2-$^2H_1$]-2-aminoethanol *O*-phosphate, was obtained as a pale yellow solid (32% yield), m.p. 162–164 °C; ν_{max}(Nujol)/cm^{-1} 3600–3200 (OH), 1150 (P=O) and 1035 (P–O–C); δ_H(270 MHz; ^2H_2O) 2.38 (3 H, s, 2-CH_3) 3.23 (1 H, t, *J* 5, N-CH), 3.93 (2 H, t, *J* 5, O-CH_2), 4.36 (2 H, s, 4′-CH_2), 4.78 (2 H, d, $J_{5'\text{-H,P}}$ 11, 5′-CH_2) and 7.62 (1 H, s, 6-H); *m*/*z* (FAB-glycerol–acetic acid matrix) 374 ([M + H]$^+$).

[1″-$^2H_2$]-N$^{4'}$-(2″*Phosphoethyl*)*pyridoxamine* 5′-*phosphate* **7**(A = ^{2}H, B = ^{2}H). This compound, prepared in a manner identical with that used for the unlabelled compound, starting from [2-$^2H_2$]-2-aminoethanol *O*-phosphate, was obtained as a pale yellow solid (33% yield), m.p. 162–164 °C; ν_{max}(Nujol)/cm^{-1} 3600–3200 (OH), 1150 (P=O) and 1035 (P–O–C); δ_H(270 MHz; ^2H_2O) 2.38 (3 H, s, 2-CH_3), 3.93 (2 H, t, *J* 5, O-CH_2), 4.36 (2 H, s, 4′-CH_2), 4.78 (2 H, d, $J_{5'\text{-H,P}}$, 5′-CH_2) and 7.62 (1 H, s, 6-H); *m*/*z* (FAB-glycerol–acetic acid matrix) 375 ([M + H]$^+$).

N$^{4'}$-(2″-*Phosphoethyl*)*pyridoxamine*.—Pyridoxal (200 mg, 1.0 mmol) was dissolved in 2 mol dm^{-3} aqueous NaOH, the solution lyophilized and the residue redissolved in dry methanol (10 cm^3). The pyridoxal salt was added in small portions to the sodium salt of 2-aminoethanol *O*-phosphate (230 mg, 1.2 mmol) in dry methanol (10 cm^3). The mixture was refluxed in the dark for 2 h, cooled to 0 °C, and treated with sodium borohydride (56 mg, 1.5 mmol), added with stirring. The resulting solution was allowed to warm to room temperature and was then acidified to pH 3 (using 6 mol dm^{-3} HCl). The solvents were removed under reduced pressure to give the crude product as a yellow solid. The solid was dissolved in 50 mmol dm^{-3} aqueous $NaHCO_3$ (100 cm^3), and the pH of the solution adjusted to 8.0 with Na_2CO_3; the solution was then purified on DEAE-Sephadex A-25-120 as described above. The UV light-absorbing fractions were pooled and reduced in volume under reduced pressure to give a pale yellow solid (150 mg, 50%), m.p. 175–177 °C; *m*/*z* (FAB) (Found: [M + H]$^+$ 293.0899. $C_{10}H_{18}N_2O_6P$ requires 293.0902); ν_{max}(Nujol)/cm^{-1} 3600–3200 (OH), 1150 (P=O) and 1035 (P–O–C); λ_{max}/nm (0.1 mmol dm^{-3} $NaOH_{aq}$) 325 (ε/dm^3 mol^{-1} cm^{-1} 10 000) and 252 (7000); δ_H(270 MHz; ^2H_2O) 2.38 (3 H, s, 2-CH_3), 3.23 (2 H, t, *J* 5, N-CH_2), 3.93 (2 H, t, *J* 5, O-CH_2), 4.36 (2 H, s, 4′-CH_2), 4.78 (2 H, d, $J_{5\text{-H,P}}$ 11, 5′-CH_2) and 7.62 (1 H, s, 6-H).

Radiochemical Assay of GAD Activity.[19]—Assay incubations containing (2*S*)-[^{14}C]glutamic acid (18 mmol dm^{-3}, *ca.* 40 000 dpm) and PLP (0.4 mmol dm^{-3}) in a total volume of 200 mm^3 of pyridine–HCl buffer (200 mmol dm^{-3}, pH 4.6) were prepared in 6 cm^3 scintillation vials and warmed to 37 °C. GAD (0.7 mg, 14 units) was dissolved in pyridine–HCl buffer (10 cm^3, 200 mmol dm^{-3}, pH 4.6). Reactions were initiated by simultaneous addition of a 200 mm^3 aliquot of protein to each incubation. The progress of the reaction was followed by terminating incubations at t = 0 and at suitable times thereafter (typically 5, 10, 15, 25 and 30 min) by the addition of 200 mm^3 of sulphuric acid (6 mol dm^{-3}). After 30 min (when the reactions were judged to have evolved all the CO_2 produced during the decarboxylation reaction) 3 cm^3 of scintillant (Optipase, Hi-safe 3) was added to the reaction mixtures. The vials were vigorously shaken to ensure complete mixing of the scintillant and incubation solution and the residual radioactivity was determined by scintillation counting. The data was processed to provide concentration *vs.* time plots which gave very shallow curves. From the tangents of the curves the initial velocity of the reaction was estimated and the enzyme activity was determined.

Unit definition. A unit of activity is that amount of protein which catalyses the production of 1 μmol min^{-1} of CO_2 under the above assay conditions.

Preparation of E. coli *GAD Apoenzyme*.[21]—*E. coli* GAD (1.5 mg, 30 units) was dissolved in pyridine–HCl buffer (5 cm^3, 200 mmol dm^{-3}, pH 4.6). α-Methyl-(±)-glutamate (80 mg, 0.5 mmol) was added slowly to the solution with gentle stirring, and the pH was maintained at 4.6 throughout by addition of potassium phosphate buffer (1 mol dm^{-3}, pH 7.0). The protein was dialysed over 16 h against several changes of piperazine–HCl buffer (100 mmol dm^{-3}, pH 4.6) containing dithiothretiol (0.1 mmol dm^{-3}) and glycerol (10% v/v). The resulting apoenzyme was assayed for protein activity in both the presence and absence of exogenous PLP. Typically the preparation in the absence of PLP showed 2% activity of that in the presence of PLP.

Incubation of E. coli *GAD with* N$^{4'}$-(2″-*Phosphoethyl*)*pyridoxamine* 5′-*Phosphate* **7**.—Five identical solutions of *E. coli* GAD apoenzyme (5.2 μmol dm^{-3}) in piperazine buffer (0.5 cm^3, 100 mmol dm^{-3}, pH 4.6) containing dithiothretiol (0.1 mmol dm^{-3}) and glycerol (10% v/v) were treated with 0.5 cm^3 solutions of *N*$^{4'}$-(2″-phosphoethyl)pyridoxamine 5′-phosphate **7**(A = B = H) in distilled water at five different concentrations (0, 20, 40, 200 and 1000 μmol dm^{-3}). The reactions were kept at 30 °C for exactly 180 min when duplicate 0.2 cm^3 aliquots were removed and assayed for activity using the methods described above. The entire experiment was performed in duplicate using a new batch of enzyme. The activity data was corrected by subtracting the activity of the control (no added activator) from the other activities and the apparent rate constants of activation were obtained from logarithmic plots of unactivated enzyme *vs.* time for different concentrations of the activator as described in the Results and Discussion section. Double reciprocal plots of k^{app} *vs.* activator concentration gave $1/k_{cat}$ and $-1/K_{Act}$ at the ordinate and abscissa intercepts respectively (see Table 2). The whole experiment was repeated in duplicate for each of the deuteriated activators [**7**(A = ^{2}H, B = H), **7**(A = H, B = ^{2}H), **7**(A = ^{2}H, B = ^{2}H)]. The results are shown in Table 2.

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