The Stereochemical Course of Reactions Catalysed by *Escherichia coli* Glutamic Acid Decarboxylase

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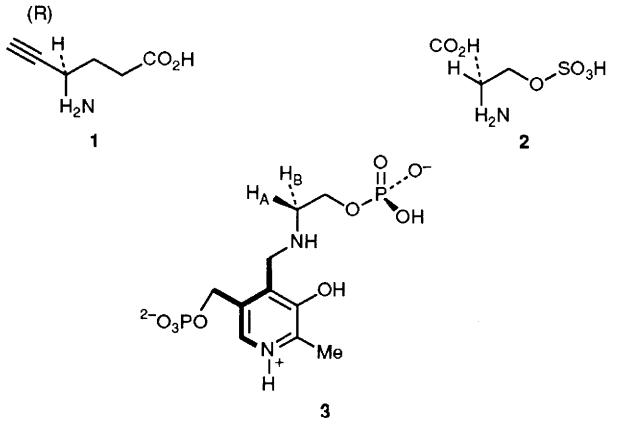
Escherischia coli glutamic acid decarboxylase reprotonates the quinoid intermediate derived from (2*S*)-glutamic acid on the 4′-*Si*-face of the coenzyme in an abortive transamination reaction, introduces the 3-*pro-R* hydrogen of β-alanine during the decarboxylation of (2*S*)-aspartic acid and removes the 1-*pro-R* hydrogen of $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5′-phosphate in a reactivation reaction; the results indicate that the distal binding groups of 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, glutamic acid decarboxylase (GAD), aromatic amino acid decarboxylase and histidine decarboxylase.

In view of their potential as targets for chemotherapeutic agents, suicide inhibitors have been designed and prepared for almost all of these enzymes.[1] In several cases the mechanistic 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,[1] for example, for acetylenic GABA [4-aminohex-5-ynoic acid, **1**][2,3] and (2*S*)-serine *O*-sulfate **2**[4], Scheme 1 [for full details see ref. 1].

In order to define the conformations of substrates and inhibitors and, hence, the positions of 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 of proton transfer to the C-4′ position of the coenzyme during an abortive 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.[5]

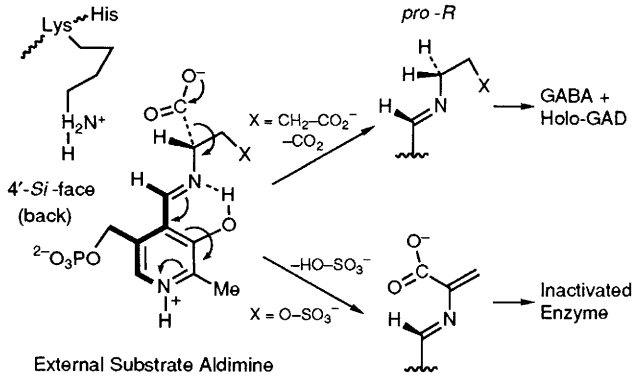
In the second, experiments were designed to determine the stereochemical course and fidelity of the decarboxylation and reprotonation reactions at C^{α} of the quinoid intermediate derived from a 'loose-fit' substrate, (2*S*)-aspartic acid. If the 'loose-fit' substrate showed the same retentive stereochemical course as for the physiological substrate[1] and, if the reaction showed high stereochemical fidelity, then it would be likely that the same conjugate acid-proton donor operated 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.[6]

In the third approach, experiments were designed to determine the stereochemical preference for a proton abstraction step in the regeneration of active holoenzyme from apoenzyme and $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5′-phosphate **3**.

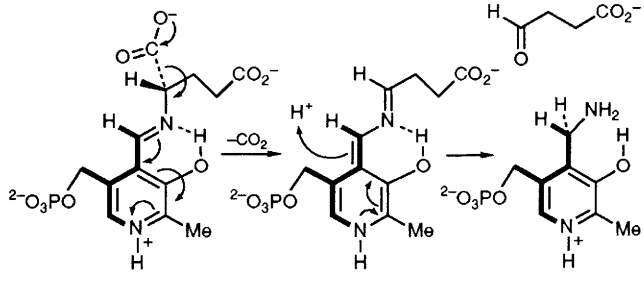
The stereospecificity of the protonation of the quinoid intermediate at C-4′ was determined using the natural substrate, (2*S*)-glutamic acid. Note, that an earlier study employed racemic 2-methylglutamic acid.[7] Unfortunately, this substrate is unsuitable for use in simple stereochemical correlations in view of its very low V_{max} value and racemic nature.

Accordingly, [4′-^{3}H]-PLP was prepared[8] and was incubated with *E. coli* GAD at pH 6.0 in the presence of (2*S*)-glutamic acid. The tritiated PMP transamination product was separated from the incubation mixture and was treated with alkaline phosphatase to give 4′-tritiated pyridoxamine. After purification, the absolute configuration at C-4′ in the sample was determined by incubation with apoaspartate aminotransferase[8] which is known to exchange the 4′-*pro-S* hydrogen of pyridoxamine with the solvent. None of the tritium was exchanged into the solvent in the sample derived from the decarboxylase incubation whereas 50% of the tritium in a racemic synthetic sample was exchanged. The result indicates that a proton is transferred to the 4′-*Si*-face of the coenzyme during the abortive transamination catalysed by the decarboxylase, Scheme 2, in accord with the results obtained for methionine decarboxylase and several transaminase enzymes.[8]

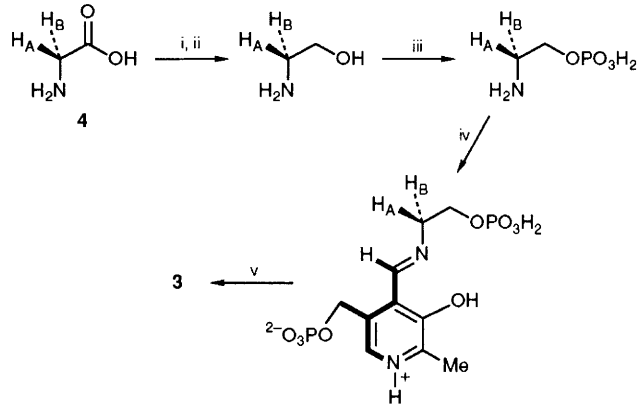
In order to determine the stereochemical course of the protonation at C^{α} in the quinoid intermediate derived from a 'loose-fit' substrate, (2*S*)[2-^{2}H]-aspartic acid and unlabelled aspartic acid were incubated with *E. coli* GAD in protium oxide and in deuterium oxide, respectively. The β-alanine products were purified on Dowex 1 formate and were each converted to their *N*-(1*S*,4*R*)-camphanamide derivatives. Analysis of the NMR and mass spectra of the derivatives and comparison of the NMR spectra with those obtained for synthetic C-3 chirally deuteriated samples and reported in the



Scheme 1



Scheme 2



Scheme 3 (Glycines prepared according to ref. 9); 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, $NaBH_4$, 0 °C, 10 min, then Sephadex A-25 cation exchange chromatography, 30% over iv and v

literature[9] indicated that the decarboxylation reaction occurred stereospecifically and with retention of configuration at C^{α} of the substrate. The high chiral integrity of the decarboxylation products† was in keeping with the notion that either a single acid or two acids operate on the 4′-*Si*-face of the coenzyme. The result mirrors those obtained for methionine decarboxylase.[6]

† The 500 MHz ^{1}H NMR spectra for the camphanamide derivative of the unlabelled compound showed well-resolved AB multiplets at δ 3.58 and 3.64. The sample derived from the incubation of C-2 deuteriated aspartic acid in protium oxide showed only a quartet at δ 3.58 while the sample derived from the incubation of unlabelled aspartic acid in deuterium oxide showed a quartet at δ 3.63 and the presence of some unlabelled material (as expected). The chemical shifts correspond to those reported for the 3-*pro-S* and 3-*pro-R* deuteriated β-alanine derivatives, respectively.[9]

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] Hence, $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5′-phosphate was prepared, through borohydride reduction of the aldimine formed from PLP and 2-aminoethyl phosphate,[10] and was incubated with *E. coli* apoGAD. 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 at pH 4.6. Active enzyme was slowly generated and in this respect the bacterial enzyme was similar to the mammalian enzyme. By repeating the experiment at a range of $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5′-phosphate concentrations the values of k_{cat} and k_m for the reactivation reaction were determined to be 1.6×10^{-4} s^{-1} and 50 μmol dm^{-3}, respectively.

In order to determine the stereochemical course of the proton abstraction step in the elimination reaction, C-2 deuteriated isotopomers (**3**, $H_A = H_B = {}^2H$; $H_A = {}^2H$, $H_B = H$, and; $H_A = H$, $H_B = {}^2H$) were prepared from the appropriate deuteriated glycines[9] according to Scheme 3.‡

When the dideuteriated compound (**3**, $H_A = H_B = {}^2H$) was incubated with *E. coli* apoGAD it was apparent that active enzyme was generated more slowly than for the unlabelled compound. The values of k_{cat} and K_m were 0.91×10^{-4} s^{-1} and 45 μmol dm^{-3}, respectively and thus, the primary deuterium isotope effect for k_{cat} was *ca.* 1.7.

Incubation of (2*S*)[2-$^2H_1$]-$N^{4'}$-(2-phosphoethyl)-pyridoxamine 5′-phosphate (**3**, $H_A = {}^2H$, $H_B = H$) with *E. coli* apoGAD gave reactivation rates identical, within experimental error, to the values obtained for the unlabelled compound. However, the (2*R*)-antipode gave rates close to those obtained for the dideuteriated compound revealing that the enzyme removes the 2-*pro-R* hydrogen from the phosphoethyl group of $N^{4'}$-(2-phosphoethyl)-pyridoxamine 5′-phosphate **3** during the reactivation process. Notably, this hydrogen atom occupies the spatially equivalent position to the proton that is introduced into the quinoid intermediate in the formation of γ-aminobutyric acid from the natural substrate, (2*S*)-glutamic acid.

The collective results of this study indicate that enzyme catalysed proton transfers occur on the 4′-*Si*-face of the coenzyme at C-4′ and C$^{\alpha}$ and that the distal binding groups of substrates and substrate analogues 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 methyne protons in the derived aldimines due to 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.

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‡ Compound **3** showed the following: m.p. 160–164 °C; *m/z* [FAB, glycerol-acetic acid] (Found: $[M + H]^+$ 373.0577. Calc. for $C_{10}H_{19}N_2O_9P_2$; 373.0566); ν_{max} (cm^{-1}, Nujol) 3600 (OH), 1150(P=O), 1035(P–O–C); λ_{max} (nm, 0.1 mol dm^{-3} $NaOH_{aq}$) 325 (ε 10000), 252 (7000); δ_H (^2H_2O) 2.38 (3H, s, 2-CH_3), 3.23 (2H, t, *J* 5 Hz, N-CH_2-), 3.93 (2H, t, *J* 5 Hz, O-CH_2-), 4.36 (2H, s, 4′-CH_2-), 4.78 (2H, d, $J_{5'\text{-H, P}}$ 11 Hz, 5′-CH_2-), 7.62 (1H, s, 6-H); δ_c (^2H_2O) 17.71 (2-CH_3), 47.01 (N-CH_2-), 50.49 (O-CH_2-), 61.99 (5′-CH_2-), 63.91 (4′-CH_2-), 126.8 (6-CH), 133.27, 137.19, 147.52 and 165.25 (2,3,4,5 and 6-C). Deuteriated isotopomers showed the expected spectral omissions and differences.

References

1 D. Gani, *Pyridoxal dependent systems, Section 6.5* in *Comprehensive Medicinal Chemistry*, ed. P. G. Sammes, Pergamon Press, Oxford, 1990, vol. 2, pp. 213–254.
2 M. Bouclier, M. J. Jung and B. Lippert, *Eur. J. Biochem.*, 1979, **98**, 363.
3 C. Danzin, N. Claverie and M. J. Jung, *Biochem. Pharmacol.*, 1984, **33**, 1741.
4 J. J. Likos, H. Ueno, R. W. Feldhaus and D. E. Metzler, *Biochemistry*, 1982, **21**, 4377.
5 D. Gani, *Philosophical Trans. (B)*, 1991, **332**, 131.
6 M. Akhtar, D. E. Stevenson and D. Gani, *Biochemistry*, 1990, **29**, 7648.
7 B. S. Sukhareva and A. E. Braunstein, *J. Mol. Biol. SSR*, 1971, **51**, 302.
8 D. E. Stevenson, M. Akhtar and D. Gani, *Biochemistry*, 1990, **29**, 7631.
9 D. Gani and D. W. Young, *J. Chem. Soc. Perkin Trans. 1*, 1985, 1355.
10 S. Y. Choi and J. E. Churchich, *Eur. J. Biochem*, 1986, **160**, 515.
11 D. Gani, Enzyme Chemistry, *Ann. Rep. Progr. Chem.*, 1986 (*B*), **83**, pp. 303–330.