# THE STEREOCHEMISTRY OF THE ABORTIVE TRANSMINATION SHOWN BY GLUTAMATE DECARBOXYLASE

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## 1. Introduction

A number of studies have begun to describe the stereochemistry of reactions catalyzed by pyridoxal phosphate (pyridoxal-P)-dependent enzymes [1]. One of these reactions, enzymatic transamination, involves reversible protonation of the cofactor $C_4'$, carbon during the key step of the reaction. The absolute stereochemistry of this protonation has been determined for three different enzymes: glutamate aspartate transaminase [2], pyridoxamine pyruvate transaminase [3] and a dialkyl amino acid transaminase [4]. In all three cases the proton is added to $C_4'$, from the '*si*' side, yielding pyridoxamine phosphate (pyridoxamine-P) with the labile proton in the pro *S*, configuration [5].

Comparisons of the $C_4'$, symmetry are thus far limited to transaminases. However, several other pyridoxal-P-dependent enzymes can be converted to their pyridoxamine-P forms by atypical transamination with appropriate substrates [6–8] and are thus available for such stereochemical studies. Of these, L-glutamate decarboxylase undergoes slow inactivation by transamination during decarboxylation of the pseudosubstrate α-methylglutamate [8]. This inactivation also occurs, but much more slowly, with the normal substrate L-glutamate [9].

An 'error' of protonation (fig. 1, path B) is presumably responsible for the transamination. This paper describes the symmetry of the decarboxylation–transamination shown by L-glutamate decarboxylase acting on α-methyl glutamate.

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## 2. Materials and methods

Glutamate decarboxylase from *E. coli* 600 was prepared by a slight modification [9] of the method of Schukuya and Schwert [10]. Our enzyme preparation was 90% pure by spectral criteria. Glutamate aspartate transaminase (GAT) was kindly supplied by A.L. Bocharov. It had been prepared from pig heart by the procedure of Polyanovsky [11] and was used as a mixture of subforms. The holoenzyme was resolved by the method of Wada and Snell [12] and the apoenzyme assayed under conditions described by Dunathan et al. [2].

Pyridoxal-P, pyridoxamine-P and α-methyl-DL-glutamate were obtained from Sigma, and α-ketoglutarate from Reanal (Hungary). Ion exchange chromatography used Dowex 50 × 8 and Dowex 1 × 8.

Paper electrophoresis was carried out on Whatman 3 MM paper. Pyridoxamine-P was purified by electrophoresis for 1 hr at 70 V/cm in a buffer of pyridine–acetic acid–water (1:10:189, v/v) at pH 3.5. Pyridoxal Pyridoxal-P was purified by electrophoresis in a pH 1.9 buffer of acetic acid–formic acid (85%)–water (4:1:45, v/v) at 100 V/cm for 45 min.

All counting was done using an Intertechnique Liquid Scintillation Spectrometer, model SL-40. 15 ml of a dioxane–naphthalene solution of 0.4% PPO and 0.02% POPOP with 0.1 ml of the aqueous phase under study was counted. All samples were corrected for quenching effects.

## 3. Results

In a preliminary experiment 8 mg glutamate decarboxylase was incubated with a five-fold excess of pyridoxal-P in a standard reaction mixture containing 160 μmoles α-methyl-DL-glutamate and $2 \times 10^{-3}$ M EDTA in 0.1 pyridine-HCl buffer, pH 4.6. The pyridoxal-P was completely converted to pyridoxamine-P during 20 hr at 25°. The conversion was followed spectrophotometrically and the pyridoxamine-P produced was isolated. Thus, the rates of decarboxylation–abortive transamination, dissociation of pyridoxamine-P and binding of pyridoxal-P are sufficiently rapid for the preparation of quantities of pyridoxamine-P greatly in excess of the quantity of enzyme active site.

When pyridoxal-P, 0.8 μmole, was incubated in this reaction mixture without enzyme for 21 hr at 25°, about 5% of the pyridoxal-P was lost via non-enzymatic reactions. The extent of exchange of $C'_4$, hydrogen in the absence of enzyme was tested by incubating 0.8 μmole of both pyridoxal-P and pyridoxamine-P in 1.0 ml of the reaction mixture containing 5.4 mCi of $^3H_2O$. Incorporation of $^3H$ into pyridoxamine-P was less than 3% of that expected for complete exchange of one hydrogen atom.

Asymmetrically tritiated pyridoxamine-P was prepared by decarboxylation–transamination of 8.0 mg of glutamate decarboxylase (0.16 μmole of active site) with 160 μmoles α-methylglutamate and 0.8 μmole of added pyridoxal-P in 1.0 ml of the reaction mixture containing 60 mCi of $^3H_2O$ and EDTA (see above). After 20 hr at 25° the enzyme was precipitated with ethanol, the supernatant and washes applied to a Dowex 50 × 8 column in washed acid form, and the column washed with water until the $^3H$ counts in the eluate approached background. The pyridoxamine-P along with some α-methyl glutamate was eluted with 0.1 N ammonia. This solution was lyophilized and the pyridoxamine-P purified by paper electrophoresis.

This purified pyridoxamine-P $^3H$ was used to reactivate somewhat more than an equivalent amount of apo-GAT in the presence of α-ketoglutarate. The reactivation was followed both spectrophotometrically and by loss of the ability of the apoenzyme to transaminate pyridoxamine with ketoglutarate [3]. When reactivation was complete, the mixture of holo- and apo-GAT was precipitated with ammonium sulfate. The supernatant and washings were counted to determine $^3H$ released as $^3H_2O$, and the precipitated holoenzyme was resolved in 60% ammonium sulfate at pH 2.6. The supernatant from this solution was counted to determine $^3H$ in pyridoxal-P.

Table 1
The release of $^3H$ from $^3H$-pyridoxamine-P when various samples of the $^3H$-PMP are bound to apo-GAT and converted into bound pyridoxal-P by transamination with ketoglutarate.

| Exp. | $^3H$-PMP (cpm/μM) | $^3H$-PMP (μM) | Holo-GAT formed[c] (μM) | $^3H$ released in $H_2O$[d] (cps) | $^3H$ remaining in holoenzyme[d] (cps) | % $^3H$ released |
|---|---|---|---|---|---|---|
| A | 4690[b] (4000)[a] | 0.30[b] | 0.11 | 288 | 42 | 87 |
| B | 2650 (4000)[a] | 0.21 | 0.21 | 550 | 56 | 91 |
| C | 330 (1400)[a] | 0.19 | 0.16 | 4 | 46 | 11 |

In experiments A and B the $^3H$-pyridoxamine-P was produced from pyridoxal-P during the decarboxylation–transamination of α-methyl glutamate decarboxylase in $^3H_2O$. In experiment C the starting pyridoxal-P contained $^3H$ at $C_4$, position and the decarboxylation–transamination was carried out in $H_2O$.

[a] In parentheses, cps expected for incorporation of one proton in absence of isotope effects.
[b] A loss in the final step of $^3H$-pyridoxamine-P isolation made this figure inaccurate.
[c] Calculated from loss in apo-GAT activity (see Methods).
[d] Counting error was < 6% in A and B and < 10% in C.

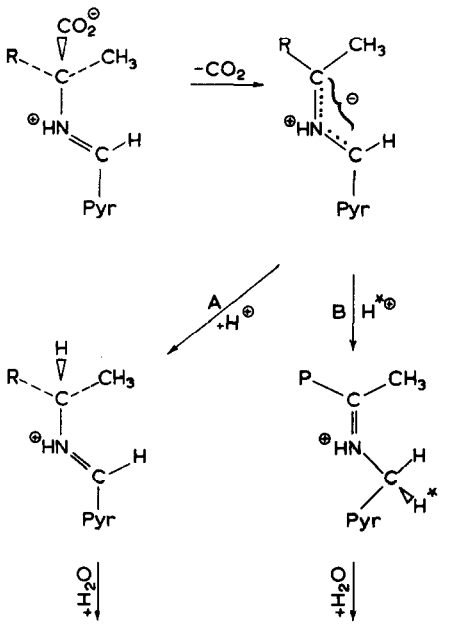


Fig. 1. Scheme of the reactions of α-methyl glutamate in the active center of glutamate decarboxylase [after 8]. (A) Pathway for normal decarboxylation reaction, (B) pathway for decarboxylation associated with abortive transamination.

R = $-CH_2CH_2COOH$; Pyr = $H_2O_3POCH_2$

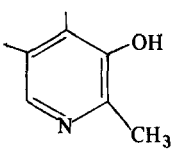

Table 1, A and B shows the results of two such experiments. The $^3H$ added at $C_4$, during decarboxylation–transamination is almost completely removed during reverse transamination of pyridoxamine-P to pyridoxal-P on binding to apo-GAT.

The minor image experiment was carried out with pyridoxal-P tritiated in the $C_4$, position which was prepared by incubation of pyridoxal-P and pyridoxamine-P at pH 6.0 in an unbuffered solution containing 22.5 mCi of $^3H_2O$. Under these conditions the non-phosphorylated pyridoxamine and pyridoxal undergo $C_4'$, exchange, presumably by non-enzymatic transamination [2]. The pyridoxal-P, pyridoxamine-P mixture also exchanges $C_4'$, hydrogen but, as with the non-phosphorylated compounds, considerable pyridoxal-P

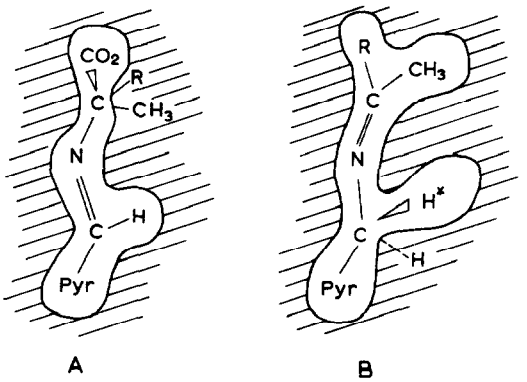


Fig. 2. The bound cofactor–substrate complex of glutamate decarboxylase before decarboxylation (A) and after the 'error' of protonation at $C_4$ (B). The exposed '*si*' side of $C_4$ in A is protonated, after loss of $CO_2$, in the abortive pathway to give the ketimine B with the newly added proton H* in the pro *S* configuration.

is lost by carbon–carbon condensation reactions. The mixture of pyridoxal-P and pyridoxamine-P was separated on Dowex 1, acetate form, and the crude pyridoxal-P further purified by electrophoresis. This $^3H$-pyridoxal-P was converted into $^3H$-pyridoxamine-P by the enzymatic decarboxylation–transamination reaction in unlabeled water. Table 1 C shows the fate of this label in the reaction with apo-GAT. Pyridoxal-P already labeled in the $C_4'$, hydrogen goes through the decarboxylation–transamination and transamination steps with little loss of label.

Thus, the symmetry of both enzymatic reactions at $C_4'$, is the same. In the abortive decarboxylation–transamination reaction of glutamate decarboxylase the $C_4'$, carbon of the intermediate anion is protonated on the '*si*' face [5] yielding pyridoxamine-P with the newly added proton of pro *S* symmetry (see fig. 2).

## 4. Discussion

In normal enzymatic α-decarboxylation we assume that loss of the $CO_2$ takes place from the exposed face of the cofactor–substrate complex and in a plane perpendicular to that of the cofactor *pi* system [13]. In one case it has been shown that the protonation of the amino acid $C_\alpha$ after loss of $CO_2$ occurs on the side from which $CO_2$ was expelled [14].

We find it reasonable to assume that the abnormal protonation of $C_4'$, also occurs from the exposed side, as shown in fig. 2. Since the added proton at $C_4'$, has pro *S* symmetry, it follows that the '*si*' face of $C_4'$, shown is in fact the exposed face. This is the side that is protonated (and thus we assume 'exposed') in each of the three cases already known [2–4]. As argued in reference [13] bond making and breaking at $C_\alpha$ (or $C_4'$) should take place in a plane perpendicular to the plane of the cofactor pi system. These arguments cannot, of course, predict which side of the cofactor pi system is involved. The four enzymes so far examined represent three quite different reaction types yet all protonate $C_4'$ from the same face. This suggests that these (and perhaps all) pyridoxal-P enzymes have similar cofactor binding sites, and that the required $C_\alpha$–N conformations for different reactions are achieved in different enzymes by changes in the geometry of substrate binding sites relative to a bound cofactor of constant geometry.

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## References

[1] H.C. Dunathan, Advan. Enzymology 34 (1971) in press.
[2] H.C. Dunathan, L. Davis, P.G. Kury and M. Kaplan, Biochemistry 7 (1968) 4532; P. Besmer and D. Arigoni, Chimia (Switz.) 23 (1969) 190.
[3] J.E. Ayling, H.C. Dunathan and E.E. Snell, Biochemistry 7 (1968) 4537.
[4] G.B. Bailey, T. Kusamrarn and K. Vuttivej, Federation Proc. 29 (1970) 857.
[5] K.R. Hanson, J. Am. Chem. Soc. 88 (1966) 2731.
[6] A. Novogrodsky, J.S. Nishimura and A. Meister, J. Biol. Chem. 238 (1963) 1903.
[7] L. Schirch and W.T. Jenkins, J. Biol. Chem. 239 (1964) 3801.
[8] T.E. Huntley and D. Metzler, in Symposium on Pyridoxal Enzymes, eds. K. Yamada, N. Katunuma and H. Wada, (Maruzen, Tokyo, 1968) p. 81.
[9] B.S. Sukhareva and A.E. Braunstein, Molekul. Biol. SSSR 5 (1971) 302.
[10] R. Schukuya and R. Schwert, J. Biol. Chem. 235 (1960) 1649.
[11] O.L. Polyanovsky, Biokhimiya 27 (1962) 734.
[12] H. Wada and E.E. Snell, J. Biol. Chem. 237 (1962) 127.
[13] H.C. Dunathan, Proc. Natl. Acad. Sci. U.S. 55 (1966) 712.
[14] B. Belleau and J. Burba, J. Am. Chem. Soc. 82 (1960) 5751.