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DEUTERIUM ISOTOPE EFFECTS IN ENZYMATIC TRANSAMINATION

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SUMMARY

The ionic fraction from the hydrolysate of residual cell walls of *Scenedesmus obliquus* grown in a medium containing 99.6% ^2H_2O was subjected to ion-exchange chromatography for the fractionation of fully deuterated L-glutamic acid. L-[2-^{2}H]-Glutamic acid and L-[3,3,4,4-$^2H_4$]glutamic acid were synthesized from L-glutamic acid and L-[2,3,3,4,4-$^2H_5$]glutamic acid, respectively.

A kinetic study of enzymatic transamination was conducted using the four L-glutamic acid analogs (L-glutamic acid and three deuterated analogs) as the substrates. The rate of transamination was followed by photometric measurements at 280 mμ. Maximum velocity determinations, based on the binary mechanism of the enzymatic transamination, were performed on each of the L-glutamic acid analogs. The deuterium isotope effect reflected in the rate of transamination was estimated through maximum velocity analysis.

The compound with deuterium in the α-position showed an isotope effect of 1.85. A similar isotope effect was observed for the compound with deuterium at the α-, β- and γ-carbons. The data indicate that the bond to the α-carbon and not to the β-carbon is ruptured during transamination. It is clearly demonstrated, therefore, that an α-elimination mechanism is involved in the enzymatic transamination.

INTRODUCTION

OSHIMA AND TAMIYA[1] reported that during enzymatic transamination between alanine and α-ketoglutaric acid in solvent ^2H_2O, hydrogen atoms at both the α- and β-carbons of alanine exchanged with deuterium atoms of the medium at similar rates. Apparently the intermediate Schiff base loses a proton from the β-carbon, a mechanism which is contrary to the prevailing notion that a proton is lost only from the α-carbon atom[2–4]. It was suggested that dissociation from the β-carbon is facilitated by formation of a hyperconjugated double-bond system in the proposed intermediate.

Incorporation of deuterium from solvent heavy water into the substrate during transamination has been reported by a number of workers[5–9]. In each case the deu-

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terium atom was assigned unequivocally to the α-carbon. HERBST AND RITTENBERG[10], however, confirmed the findings of OSHIMA AND TAMIYA[1] by postulating that α- and β-hydrogen atoms of alanine exchanged with deuterium of the medium when pyruvic acid and α-aminophenylacetic acid were used as reactants.

HARLEY-MASON[11] has criticized the proposal of OSHIMA AND TAMIYA[1] as involving improbable structures and points out that β-hydrogen exchanging with deuterium in the medium does not prove that β-elimination is a prime step in transamination. The carbonyl and the α-cyano group in the Schiff base intermediate promote nonenzymatic exchange of the hydrogen at the β-carbon and therefore would be an expected concomitant of the transamination process.

Because of enolization of α-keto acids in an aqueous medium, the determination of the exact mechanism by direct substrate analysis becomes complicated. The present investigation was designed to study this problem. A series of deuterated analogs of glutamic acid was prepared and the deuterium isotope effect in enzymatic transamination was determined. Through this type of definitive experiment one is able to determine whether α- or β-elimination is involved in the transamination reaction, since deuterium, if present in the specific position involved in proton elimination, may elicit an isotope effect and show a measurable decrease in initial velocity.

EXPERIMENTAL

Isolation and purification of L-[2,3,3,4,4-$^2H_5$]*glutamic acid*

L-[2,3,3,4,4-$^2H_5$]Glutamic acid was isolated and purified as described earlier[12]. The NMR spectrum of the deuterio-amino acid was devoid of absorption peaks for the hydrogens at the α-, β- and γ-positions. The microanalysis data provided additional evidence for the proposed structure by agreement with the calculated values. The compound was recrystallized from water, m.p. 208–209° decomp. Analysis: Calculated for $C_5H_4{}^2H_5NO_4$: C, 39.46; H + ^2H, 9.27; N, 9.20. Found: C, 39.49; H + ^2H, 9.19; N, 9.25.

Synthesis of L-[2-^2H]*glutamic acid*

L-Glutamic acid (5 g, 34.0 mmoles) was acetylated with acetic anhydride (9 ml, 95.3 mmoles) in a basic medium. Purification through Dowex 50-X8 (H^+) column chromatography yielded *N*-acetyl-L-glutamic acid (6.0 g, 93% yield), m.p. 203–204°, $[\alpha]_D^{25}$ −16.4 (c = 2, H_2O).

N-Acetyl-L-glutamic acid (1 g) was stirred into a solution containing 4 M NaO^2H (3 ml), ^2H_2O (3 ml) and acetic anhydride (35 ml). The mixture was kept at 50° for 48 h to effect racemization. ^2H_2O (15 ml) was added and the reaction mixture was kept at 50° for an additional 30 min. The reaction mixture was then evaporated to dryness. The dry residue was dissolved in a solution containing ^2H_2O (3 ml) and acetic anhydride (35 ml). The reaction mixture was kept at 50° for 48 h. Excess acetic anhydride was decomposed with ^2H_2O (15 ml) and the solution was evaporated to dryness. The residue was dissolved in water and passed through a Dowex 50-X8 column to effect the isolation of *N*-acetyl-DL-[2-^2H]glutamic acid (0.8 g, 80% yield), m.p. 186–188°. Complete deuteration at the α-position was established by the absence of a peak in its NMR spectrum corresponding to the α-hydrogen atom of *N*-acetyl-DL-glutamic-acid.

The L-enantiomorph of *N*-acetyl-DL-glutamic acid (0.52 g, 2.7 mmoles) was selectively hydrolyzed by treating an aqueous solution at pH 7.2 with acylase (15 mg). The solution was kept at 37° for 3 h. Fresh acylase (15 mg) was added, and the reaction was continued for 2 h. The enzyme was then removed by treatment with charcoal and the reaction mixture was passed through a Dowex 50-X8 column. Free amino acid retained by the column was eluted with 5% NH_4OH. The effluent containing L-glutamic acid was treated with charcoal, concentrated and chilled. The white crystalline residue was recrystallized from water to yield L-[2-^{2}H]glutamic acid (0.18 g, 90% yield), m.p. 209–210°. Its NMR spectrum showed no peak corresponding to the α-hydrogen atom of L-glutamic acid. Analysis: Calculated for $C_5H_8{}^2HNO_4$: C, 40.54; H + ^{2}H, 6.80; N, 9.46. Found: C, 40.40; H + ^{2}H, 6.84; N, 9.66.

Synthesis of L-[*3,3,4,4-*$^2H_4$]*glutamic acid*

L-[2,3,3,4,4-$^2H_5$]Glutamic acid (0.37 g, 2.4 mmoles) was reacted with acetic anhydride (0.7 ml, 7.4 mmoles) to yield *N*-acetyl-L-[2,3,3,4,4-$^2H_5$]glutamic acid (0.4 g, 85% yield), m.p. 204–205°.

The racemization procedure was the same as that described for *N*-acetyl-L-glutamic acid, except that 4 M NaOH and H_2O were used in place of 4 M NaO^2H and ^2H_2O, respectively. *N*-Acetyl-L-[2,3,3,4,4-$^2H_5$]glutamic acid (400 mg) was converted to *N*-acetyl-DL-[3,3,4,4-$^2H_4$]glutamic acid (300 mg, 75% yield), m.p. 188–190°. The NMR spectrum showed a singlet peak corresponding to the α-hydrogen of *N*-acetyl-DL-glutamic acid. Complete protiation at the α-position was established by the fact that the integral value of the peak corresponding to the α-hydrogen was exactly one third of the peak corresponding to the three hydrogen atoms of the acetyl group.

N-Acetyl-DL-[3,3,4,4-$^2H_4$]glutamic acid (0.22 g, 1.2 mmoles) was selectively hydrolyzed to yield L-[3,3,4,4-$^2H_4$]glutamic acid (0.073 g, 85% yield), m.p. 201–202°. Analysis: Calculated for $C_5H_5{}^2H_4NO_4$: C, 39.73; H+^{2}H, 8.66; N, 9.26. Found: C, 39.04; H + ^{2}H, 8.28; N, 9.42.

Transamination study

The rate of transamination was followed by measuring the absorbance of oxaloacetic acid at 280 mμ, using a Zeiss PMQ II spectrophotometer. Glutamate–oxaloacetate aminotransferase obtained from Mann Research Laboratory was dialyzed overnight against imidazole–HCl buffer solution (0.1 M, pH 7.38), which served as the buffer in the transamination reaction mixtures.

In general, the following procedure was used: To a 5-cm cuvette containing appropriate quantities of amino acid and oxaloacetic acid stock solutions, sufficient buffer solution was added to give a total volume of 7.9 ml. The reaction was initiated by the addition of 0.1 ml of enzyme preparation. The absorbances were recorded every 10 sec. Velocity data were derived from the slope of the initial linear portion of the reaction curve obtained when the absorbance readings were plotted against time.

RESULTS AND DISCUSSION

Synthesis of partially deuterated glutamic acids

The forms of L-glutamic acid used for these studies included: (1) L-glutamic

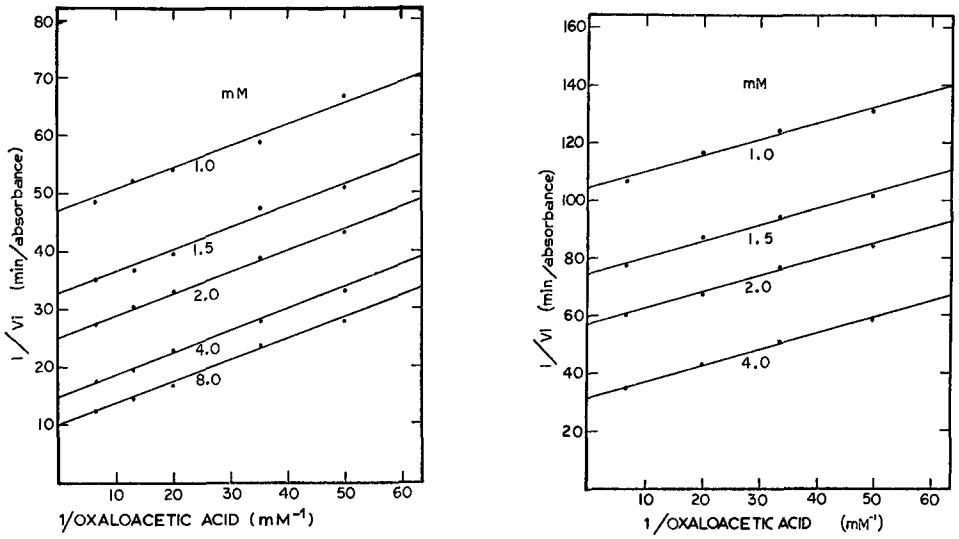


Fig. 1. Double-reciprocal plot of initial velocities against oxaloacetic acid concentration at a series of fixed concentrations (mM) of L-glutamic acid indicated at the curves (0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml).

Fig. 2. Double-reciprocal plot of initial velocities against oxaloacetic acid concentration at a series of fixed concentrations (mM) of L-[3,3,4,4-$^2H_4$]glutamic acid indicated at the curves (0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml).

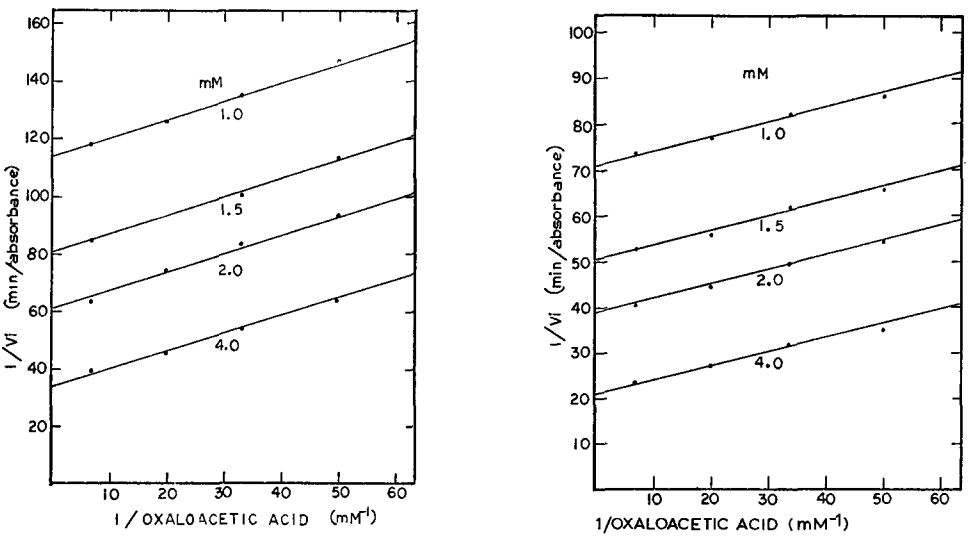


Fig. 3. Double-reciprocal plot of initial velocities against oxaloacetic acid concentration at a series of fixed concentrations (mM) of L-[2-^2H]glutamic acid indicated at the curves (0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml).

Fig. 4. Double-reciprocal plot of initial velocities against oxaloacetic acid concentration at a series of fixed concentrations (mM) of L-[2,3,3,4,4-$^2H_5$]glutamic acid indicated at the curves (0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml).

acid, (2) L-[2-^{2}H]glutamic acid, (3) L-[2,3,3,4,4-$^2H_5$]glutamic acid and (4) L-[3,3,4,4-$^2H_4$]glutamic acid.

Compound 1 was obtained from commercial sources. Compound 3 was obtained from a biosynthetic process involving the cultivation of algae in a medium containing 99.6% ^2H_2O. Compounds 2 and 4 were prepared from Compounds 1 and 3, respectively, through a racemization and selective hydrolysis procedure. The overall synthesis involved three steps: (1) acetylation, (2) racemization and (3) enzymatic hydrolysis to give the free glutamic acid. An overall yield of 55% was attained, based on 0.37 g of starting compound.

In the racemization step acetylglutamic acid is first converted to the azlactone by heating with acetic anhydride in the presence of sodium acetate. The subsequent tautomerization of the azlactone results in the racemization of the amino acid. Since the α-hydrogen atom (or deuterium atom) of the amino acid exchanges freely with the deuterium atom (or hydrogen atom) of the medium in the tautomerization process, the substitution of either deuterium or hydrogen at the α-position will, therefore, be quantitatively achieved by using either ^2H_2O or H_2O as the racemization medium. Complete substitution at the α-position may be achieved by repeating the racemization reaction with fresh ^2H_2O or H_2O. The extent of deuterium enrichment in each compound was determined through analysis of the NMR spectra.

Transamination study

The steady-state kinetics of transamination, based on the binary mechanism, is represented by equation:

$$\frac{1}{v_i} = \frac{1}{v_{max} \cdot [E]} \cdot \left(1 + \frac{K_{am}}{[am]} + \frac{K_{ket}}{[ket]}\right)$$

With v_i, initial velocity; v_{max}, maximum velocity; $[E]$, enzyme concentration; K_{am}, Michaelis parameter for the amino acid; [am], amino acid concentration; [ket], keto acid concentration; and K_{ket}, Michaelis parameter for the keto acid.

The values for K_{am}, K_{ket}, and v_{max} $[E]$ were obtained from the analysis of the graphical data, shown in Figs. 1–6. The values given in Table I were obtained from the best-fit straight lines derived from "least square" regression analysis of the data.

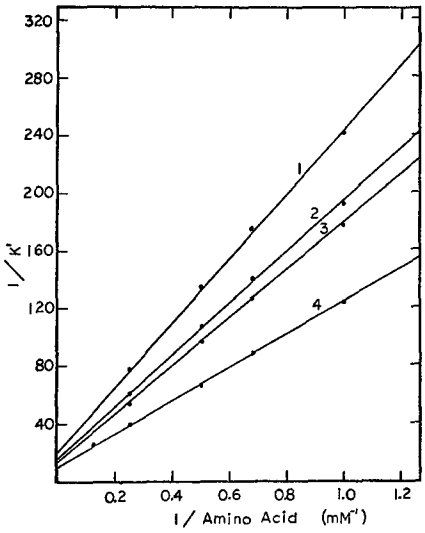
TABLE I

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES OBTAINED FROM THE TRANSAMINATION REACTIONS BETWEEN OXALOACETIC ACID AND L-GLUTAMIC ACID ANALOGS

0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml

Amino acid substrate	K_{am} *(mM)**	K_{ox} *(mM)***	v_{max} *(E)****	$\frac{v_H}{v^2_H}$
L-Glutamic acid	10.16	0.09	0.24	1.00
L-[3,3,4,4-$^2H_4$]Glutamic acid	12.68	0.06	0.19	1.26
L-[2-^{2}H]Glutamic acid	12.70	0.07	0.13	1.85
L-[2,3,3,4,4-$^2H_5$]Glutamic acid	13.44	0.08	0.13	1.85

* Michaelis constants of the amino acids.
** Michaelis constants of oxaloacetic acid.
*** Maximum velocities at the enzyme concentration of 0.094 μg/ml.



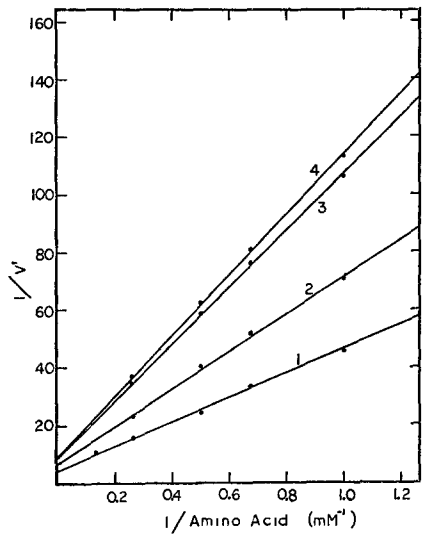


Fig. 5. Double-reciprocal plot of apparent Michaelis constants of oxaloacetic acid against concentration of L-glutamic acid and its analogs. (0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml). Curve 1, L-[3,3,4,4-$^2H_4$]glutamic acid; Curve 2, L-[2-^2H]glutamic acid; Curve 3, L-[2,3,3,4,4-$^2H_5$]glutamic acid; Curve 4, L-glutamic acid.

Fig. 6. Double-reciprocal plot of apparent maximum velocities against amino acid concentrations of L-glutamic acid and its analogs. (0.1 M imidazole buffer (pH 7.38), temp. 25°, enzyme concn. 0.094 μg/ml). Curve 1, L-glutamic acid; Curve 2, L-[3,3,4,4-$^2H_4$]glutamic acid; Curve 3, L-[2-^2H]-glutamic acid; Curve 4, L-[2,3,3,4,4-$^2H_5$]glutamic acid.

The maximum velocity of L-[2-^2H]glutamic acid is found to be 54% of that of L-glutamic acid. Since the only deuterium atom present in the molecule is the one in the α-position, the isotope effect observed in this case can only be accounted for by the α-elimination during the transamination reaction. A decrease of 32% in maximum velocity, due solely to the addition of an α-deuterium atom, is observed when the maximum velocities of L-[3,3,4,4-$^2H_4$]glutamic acid and L-[2,3,3,4,4-$^2H_5$]glutamic acid are compared. These observations indicate clearly the participation of α-elimination. The magnitude of maximum velocities for L-[2,3,3,4,4-$^2H_5$]glutamic acid and L-[2-^2H]glutamic acid is the same. These findings exclude conclusively the possibility of a single β-elimination process in the enzymatic transamination.

L-[3,3,4,4-$^2H_4$]Glutamic acid, however, shows a decrease of 25% in maximum velocity. This is explained in terms of the secondary isotope effect, where the presence of deuterium atoms in the β- and γ-positions of the molecule exert an overall effect of decreasing the rate of α-hydrogen elimination, the rate-limiting step of the reaction. The maximum velocity will, therefore, be decreased without breaking C–^2H bonds in the β-position.

The secondary isotope effect caused by the presence of deuterium atoms in the molecule on its rate of combination and dissociation with the enzyme does not affect the maximum velocity. The magnitude of this kind of secondary isotope effect will not exceed that of the rate-limiting step, involving the tautomerism of the Schiff base where α-elimination takes place.

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