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The reaction of ornithine aminotransferase with ornithine

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Rat liver ornithine aminotransferase is found to exchange the *pro-S* hydrogen on the δ-carbon atom of ornithine exclusively, thus showing that the mammalian enzyme transfers the δ-amino group and not the α-amino group as has been demonstrated with the plant enzyme [Mestichelli, Gupta & Spenser (1979) *J. Biol. Chem.* **254**, 640–647]. The enzyme also transfers the α-amino group of glutamate and the kinetics of the half reactions between the enzyme and both amino acids are compared. Rate and dissociation constants for both half reactions are determined.

Accounts of the metabolism of ornithine in standard textbooks (e.g. Mahler & Cordes, 1966; Lehninger, 1975) indicate that transamination of this diamino acid entails loss of the δ-amino group and not the α-amino group. The enzyme responsible, ornithine δ-aminotransferase (L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13), also interconverts 2-oxoglutarate and glutamate efficiently in the second half of the complete Ping-Pong mechanism. It seemed curious that although ornithine has an α-amino group configurationally identical with that of L-glutamate the enzyme should act exclusively on the δ-amino group even though at pH values near neutrality the α-amino group (pK = 8.7) is more reactive than the δ-amino group (pK = 10.8).

Mestichelli *et al.* (1979) provided evidence that the metabolic route from ornithine to proline in plants occurs via loss of the α-amino group, not the δ-amino group, and they pointed out that much of the evidence indicating δ-amino group transfer depended on the occurrence of a reaction between the product and 2-aminobenzaldehyde to give a yellow colour. The product of transamination of either group is a cyclic internal imine; Δ^1-pyrroline-5-carboxylate if the δ-amino group is transferred and Δ^1-pyrroline-2-carboxylate if the α-amino group is transferred. As pointed out by Mestichelli *et al.* (1979), both compounds react similarly with aminobenzaldehyde.

Because transamination proceeds via labilization of a proton on the relevant carbon atom, the amino group transferred may be identified by determining which proton is exchanged by the enzyme in deuterated solvent. The present paper identifies the exchangeable ornithine proton. In addition the equilibria and kinetics of the separate half reactions with ornithine and glutamate are compared.

Experimental

Enzyme preparation

Ornithine aminotransferase was prepared by a method based on that described by Peraino *et al.* (1969) but with the following modifications.

(1) The amount of the enzyme present in rat liver is increased several-fold when the animals are fed on a high-protein diet. Because of the high cost of the commercial 60% casein laboratory diet used by Peraino *et al.* (1969) the rats in the present study were fed for four days on dry textured soya bean protein (Temptein Soya Protein Meat-Like Chunks, Brooke Bond Oxo Catering Services Division, Croydon, Surrey, U.K.).

(2) The method of Peraino *et al.* (1969) includes a step in which the preparation is heated to 55°C. We experienced occasional severe losses at this stage but these were overcome by including 2-oxoglutarate (4 mM) and pyridoxal phosphate (0.2 mM) before heating. The protection afforded by these compounds allowed the enzyme to be heated to 60°C for 1 min without loss, and this treatment was used routinely.

(3) The step in which the pH is lowered briefly to 5.0 was omitted.

(4) The preparation of ornithine aminotransferase is made relatively easy because of the enzyme's unusual insolubility in $(NH_4)_2SO_4$ solutions (Matsuzawa *et al.*, 1968). Thus, after precipitation with 30% (w/v) $(NH_4)_2SO_4$, the pellet is resuspended in a small amount of buffer solution and, whereas impurities dissolve, ideally the enzyme does not. We presume that this is because there is sufficient residual $(NH_4)_2SO_4$ in the pellet to prevent the enzyme dissolving, but we found that the procedure became more reliable if at this stage the

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resuspension was carried out using buffer solution containing 1% (w/v) $(NH_4)_2SO_4$.

(5) 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) was used in all buffer solutions instead of Tris. This change was made because of a reaction that occurs between Tris and the enzyme.

Most preparations yielded enzyme that, after treatment with ornithine, lost almost all of the 422nm peak while simultaneously a peak at 330nm appeared (Fig. 1). However, occasional preparations, although homogeneous in sodium dodecyl sulphate / polyacrylamide - gel electrophoresis, retained a significant 422nm peak even at higher ornithine concentrations. The kinetic behaviour of such preparations was more complex than that reported in the Results and discussion section, there being an additional step about 50 times slower.

All the experiments described in the present paper used enzyme that showed spectral changes like those of Fig. 1.

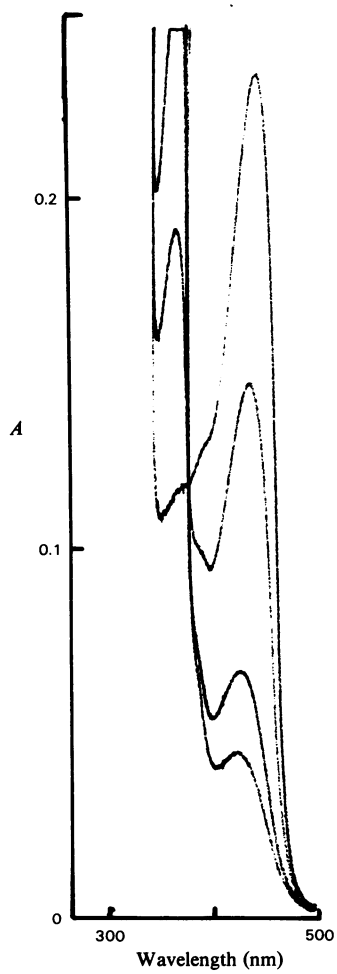

Fig. 1. *Spectral changes occurring when ornithine aminotransferase is treated with ornithine*
Aliquots (5μl) of ornithine were added successively to ornithine aminotransferase (35μM in 0.5ml of 50mM-sodium phosphate, pH8.0, at 22°C) to give final concentrations of 20, 70 and 500μM. The spectrum with the highest absorbance at 422nm is that of the untreated enzyme.

Measurement of enzyme concentration

Enzyme active site concentration was determined by measuring the change in absorbance at 412nm that occurred on treatment with 5mM-ornithine. An absorbance coefficient (6800 litre·mol^{-1}·cm^{-1}) based on the amount of pyridoxamine phosphate released from ornithine-treated enzyme in 0.5M-NaH_2PO_4 was used.

Reagents

Glutamate decarboxylase and ornithine were bought from Sigma; pyridoxal phosphate and 2-oxoglutarate were from Koch-Light. ^2H_2O (99.8%) was bought from Fluorochem, Glossop, Derbyshire, U.K. All other reagents were from BDH. 4-Aminobutyrate aminotransferase was prepared by the method of Fowler & John (1981).

Spectra

N.m.r. spectra were determined in ^2H_2O by using a Perkin–Elmer R-32 n.m.r. spectrometer. Internal standardization was achieved with 4,4-dimethyl-4-silapentane sodium sulphonate (DSS). Absorption spectra were determined with a Beckman model 25 recording spectrophotometer.

Stopped-flow measurements

Stopped-flow experiments were carried out with a Durrum Gibson stopped-flow spectrophotometer. Results were recorded by photographing the display of a Tektronix R.M.564 storage oscilloscope. The photographed traces were digitalized by projection on to an Apple (Cupertino, CA, U.S.A.) graphics tablet.

pH measurements in ^2H_2O

The pH values quoted for ^2H_2O solution are given as pH meter readings plus 0.4.

Results and discussion

Deuterium exchange catalysed by ornithine aminotransferase

The transamination mechanism requires removal of a proton from the carbon atom bearing the relevant amino group (Braunstein, 1973). If the half reaction between enzyme and amino acid is carried out in ^2H_2O the relevant proton is exchanged (Konikova *et al.*, 1947; Banks *et al.*, 1966). Fig. 2 shows n.m.r. spectra of ornithine before and after the

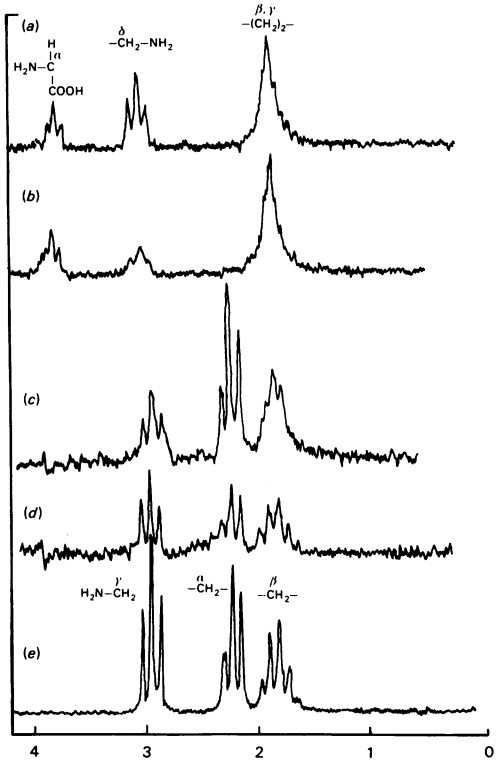

Fig. 2. *N.m.r. spectra of ornithine and its derivatives*
Spectra were determined in ^2H_2O as described in the text. (*a*) L-Ornithine, 20mg/ml; (*b*) L-ornithine after treatment for 16h with ornithine aminotransferase (15mg/ml) at 25°C and pH8.0 in ^2H_2O; (*c*) 4-amino[4-^{2}H]butyrate (10mg/ml) from oxidation of [δ-^{2}H]ornithine in (*b*); (*d*) compound derived from (*c*) after treatment with 4-aminobutyrate aminotransferase in H_2O; (*e*) authentic 4-aminobutyrate.

exchange. Within the limits of the measurement of the peak areas it seems that one of the two δ-protons is replaced exclusively. After 16h the enzyme had lost half of its activity and an equal amount of fully active enzyme was added to prolong the possibility of exchange. No further change was detectable. Because we were unable to measure any change at the α-carbon atom we can only estimate an upper limit for the rate of exchange at this position. We consider that we would clearly detect a 20% decrease in peak area and this does not occur after 32h at a mean enzyme concentration equal to that at the start. Under these conditions the δ-proton exchanged with $t_{\frac{1}{2}} = 2$h, from which we calculate that exchange of the γ-proton must be at least 50 times faster than that of the α-proton.

Stereochemistry of deuterium exchange

A sample (90mg) of ornithine was exchanged in ^2H_2O for 48h at pH7.5 using 30μM-ornithine aminotransferase. An n.m.r. spectrum showed the peak due to the protons on the δ-carbon atom to be reduced to half its area. The deuterium-labelled ornithine was separated from the enzyme on Sephadex G-25 and converted to 4-aminobutyrate by using the permanganate oxidation method of Mestichelli *et al.* (1979). The identity of the product was confirmed by t.l.c. and n.m.r. spectroscopy. The n.m.r. spectrum showed the peak due to γ-protons to be half the area of that seen in a standard 4-aminobutyrate preparation.

Two parallel experiments were performed with the 4-amino[4-^{2}H]butyrate. In the first, the deuterated compound was treated with 8μM-4-aminobutyrate aminotransferase in ^2H_2O for 48h and in the second in H_2O under the same conditions. The 4-aminobutyrate was analysed by n.m.r. in ^2H_2O after separation from the enzyme on Sephadex G-25. The 4-amino[4-$^2H_1$]butyrate remained unchanged after the ^2H_2O experiment whereas after the treatment in H_2O the peak due to the γ-protons doubled and the splitting pattern of the β-protons changed to that of normal 4-aminobutyrate. We conclude therefore that the stereochemistry of proton exchange is the same for both ornithine transaminase and 4-aminobutyrate transaminase.

To establish the absolute stereochemistry of the exchange we prepared (4*R*)-4-amino[4-$^2H_1$]butyrate by enzymic decarboxylation of glutamate in ^2H_2O (Yamada & O'Leary, 1978). Sodium glutamate (0.1M) in 0.2M-pyridine in ^2H_2O adjusted to pH4.6 with ^{2}HCl and containing 20μM-pyridoxal phosphate and 2.5 units of glutamate decarboxylase/ml was kept at 37°C for 24h. The solution was freeze-dried, dissolved in 2ml of H_2O and applied to a column (6cm × 1cm) of Dowex 1 (8% cross-linked, OH^- form) equilibrated with water. The column was eluted with water. Pyridine and 4-aminobutyrate eluted together and pyridine was removed by freeze drying. The sample was taken up in 0.5ml of ^2H_2O for n.m.r. analysis.

When a sample of this (4*R*)-4-amino[4-$^2H_1$]-butyrate was used as substrate for 4-aminobutyrate aminotransferase in ^2H_2O the n.m.r. peak assigned to the 4-proton disappeared altogether, whereas the same reaction in H_2O left the compound unchanged. Thus rabbit brain 4-aminobutyrate aminotransferase and pig kidney ornithine aminotransferase both labilize the *pro-S* proton of their respective ω-amino acid substrate. During the course of this work Bouclier *et al.* (1979) using ^3H_2O described similar experiments on the stereochemistry of pig brain 4-aminobutyrate aminotransferase, also showing labilization of the *pro-S* proton.

Equilibria of the half reactions with amino acid substrates

The half reactions may be described by eqn. (1) in which E_L and E_M are the pyridoxal and pyridoxamine forms of the enzyme, S is an amino acid substrate and P is the product of transamination.

$$E_L + S \overset{K_s}{\rightleftharpoons} E_LS \underset{k_b}{\overset{k_f}{\rightleftharpoons}} E_MP \overset{K_p}{\rightleftharpoons} E_M + P \quad (1)$$

The results described below are interpreted in terms of eqn. (1). The dissociations governed by K_s and K_p are assumed to be very rapid compared with the reactions governed by k_f and k_b. The central isomerization between E_LS and E_MP is taken to involve proton transfer from substrate to coenzyme, thereby shifting the position of the imine double bond, decreasing coenzyme resonance and producing the 420nm→330nm spectral transition (Fig. 1). In all experiments a single, first-order change was observed. A_{420}, immediately after mixing with amino acid substrates, was the same as that seen when enzyme was mixed with buffer solution alone, showing that the ε_{420} values of E_L and E_LS are not significantly different. Observed first-order rate constants ($k_{obs.}$) arising from the mechanism of eqn. (1) should depend on [S] and [P] according to eqn. (2):

$$k_{obs.} = k_f\frac{[S]}{K_s+[S]} + k_b\frac{[P]}{K_p+[P]} \quad (2)$$

The kinetics of the half reactions between E_L and both amino acid substrates were determined by measuring ΔA_{420} using a stopped-flow spectrophotometer. The reverse reaction between E_M and 2-oxoglutarate was analysed similarly. Fig. 3 shows how $k_{obs.}$ varied with ornithine concentration. At low concentrations the dependence is almost linear and extrapolation to [ornithine] = 0 gave $k_{obs.} = 0.7\,s^{-1}$. Separate equilibrium experiments showed that the relative amplitude of the spectral change observed on increasing ornithine concentration was independent of enzyme concentration in the range 10–40μM, suggesting that pyrroline-5-carboxylate binds tightly to E_M. Thus it was assumed that $k_b = 0.7\,s^{-1}$. Subtracting this value from $k_{obs.}$ obtained for each ornithine concentration and analysing the data by the method of Wilkinson (1961) gave the values of K_s and k_b shown in Table 1. The continuous line of Fig. 3 was predicted by eqn. (2) using these values and assuming a constant contribution of $0.7\,s^{-1}$ to the backward component of $k_{obs.}$

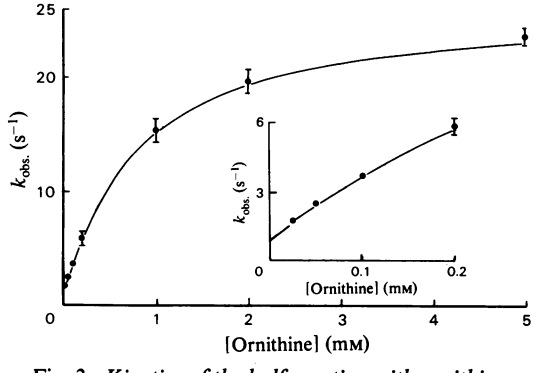

Fig. 3. *Kinetics of the half reaction with ornithine*
Ornithine aminotransferase was mixed with ornithine to give 6μM-enzyme and the glutamate concentrations shown. The reactions were carried out at 24°C in 50mM-sodium phosphate, pH 8.0.

Fig. 4 shows the results obtained for the glutamate–2-oxoglutarate half reaction analysed in both directions. When glutamate was used as the variable substrate, 2-oxoglutarate (25μM) was included so that increases in [P] from the reaction itself were insignificant. The value of $2.16\,s^{-1}$ at [glutamate] = 0 (Fig. 4*a*) is that predicted for $k_b[P]/(K_p+[P])$ when [P] = 25μM using constants arising from analysis of the half reaction between E_M and 2-oxoglutarate (Fig. 4*b*). Analysis of the data from both half reactions by the method of Wilkinson (1961) gave values for the constants shown in Table 1. The continuous lines of Fig. 4 were predicted by eqn. (2) using these constants.

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Table 1. *Kinetic constants for the half reactions between ornithine aminotransferase and amino and oxo acid substrates*
The constants were determined at 22°C, pH 8.0 in 50mM-sodium phosphate.

Half reaction	k_f (s^{-1})	k_b (s^{-1})	K_s (mM)	K_p (mM)
Ornithine/pyrroline-5-carboxylate	26.6 ± 0.4	0.7	0.87 ± 0.06	—
Glutamate/2-oxoglutarate	4.8 ± 0.5	29.6 ± 13	1.1 ± 0.3	0.32 ± 0.04

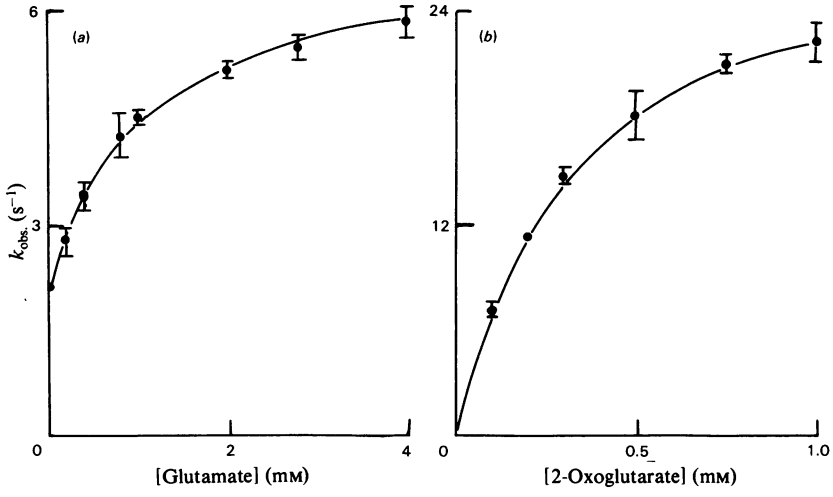

Fig. 4. *Kinetics of the half reactions with glutamate and 2-oxoglutarate*

(*a*) Ornithine aminotransferase was mixed with glutamate to give 6 μM-enzyme and the glutamate concentrations shown. The reactions were carried out at 22°C in 50 mM-sodium phosphate, pH 8.0, containing 25 μM-2-oxoglutarate. (*b*) Ornithine aminotransferase in the pyridoxamine phosphate form was mixed with 2-oxoglutarate to give 6 μM-enzyme and the substrate concentrations shown. The reactions were carried out at 22°C in 50 mM-sodium phosphate buffer, pH 8.0, containing 20 μM-glutamate.

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