Kinetic Studies with the Use of Proton-Magnetic-Resonance Spectroscopy of the Specific α -Deuteration of Amino Acids by *Escherichia coli* Aspartate Aminotransferase

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(Received 9 January 1978)

Escherichia coli aspartate aminotransferase was exposed to aspartate or phenylalanine without oxo acid in buffered $^2\text{H}_2\text{O}$. The α -hydrogen of the amino acids underwent first-order exchange with respect to both substrate and enzyme. P.m.r. spectroscopy gave consistent reaction-rate constants. The deuterium-exchange rate was only moderately increased by addition of oxo acids and was of the same order as the transamination rate. No β -deuteration was observed. The $\text{C}_{(\alpha)}\text{--H}$ -bond-breaking step is discussed as a part of the entire transamination mechanism.

The formation of a Schiff base between an α -amino acid and pyridoxal phosphate and the labilization of the α -hydrogen of the amino acid are essential steps of all pyridoxal phosphate-catalysed reactions (Braunstein, 1973; Snell & Di Mari, 1970). An isotopic incorporation in the α -position of the amino acid was observed by i.r. spectroscopy (Oshima & Tamiya, 1961), mass spectroscopy (Hilton *et al.*, 1956), ^3H exchange (Walter *et al.*, 1975) or p.m.r. spectroscopy (Walter *et al.*, 1975; Whelan & Long, 1969; Cooper, 1976; Babu & Johnston, 1976; Golichowski *et al.*, 1977) when different mammalian amino acid–oxo acid aminotransferases were studied in deuterated water. Enzyme-catalysed exchange of the β -hydrogen atoms has been reported only for alanine of the amino acids (Walter *et al.*, 1975; Cooper, 1976; Babu & Johnston, 1976; Golichowski *et al.*, 1977), and for oxaloacetate of the oxo acids by Whelan & Long (1969).

This present paper describes the deuterium exchange between aspartate or phenylalanine and $^2\text{H}_2\text{O}$ catalysed by *E. coli* aspartate aminotransferase (EC 2.6.1.1), which has a broader specificity, extending to aromatic amino acid substrates (Chesne & Pelmont, 1973; Chesne *et al.*, 1975).

Materials and Methods

Materials

L-Phenylalanine, L-aspartic acid, potassium phosphate and EDTA were purchased from Merck (Darmstadt, Germany), sodium succinate was from Boehringer (Mannheim, Germany), and 2-oxoglutarate, oxaloacetate, phenylpyruvate and pyridoxal 5'-phosphate were from Fluka (9470 Buchs, Switzerland).

All chemicals used were of reagent grade, when available. $^2\text{H}_2\text{O}$ (99.6% ^2H content) and NaO^2H (99.2%) were purchased from CEA, Saclay, France.

Purification of the enzyme

Aspartate aminotransferase was purified from *E. coli* K12 essentially by the procedure of Chesne & Pelmont (1973) without any heating step, with the following modification. After the final $(\text{NH}_4)_2\text{SO}_4$ precipitation and centrifugation, the sediment was dissolved in 100 ml of 25 mM-sodium succinate buffer, pH 5.4, containing 0.1 mM-pyridoxal 5'-phosphate and 1 mM-2-oxoglutarate, then dialysed overnight against the same buffer. Insoluble material was removed by a short centrifugation at 30000g, and the supernatant was chromatographed on a CM-Sephadex C-50 column (35 cm $\times$ 2.5 cm) (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. Aspartate aminotransferase was not retained on this column and was further purified as previously described. The purified enzyme showed only one band after polyacrylamide-gel electrophoresis, and was dialysed against 20 mM-potassium phosphate buffer, pH 6.8. It was freeze-dried and stored at -25°C before p.m.r. experiments.

Assays were performed as described by Chesne & Pelmont (1974). One unit of enzyme is defined as the quantity of enzyme transforming 1 μmol of aspartate/min in the aspartate–oxoglutarate reaction.

Kinetics of deuteration of amino acids by p.m.r. spectroscopy

The exchange reaction was conducted in an n.m.r. tube containing 100 μmol of L-phenylalanine or

Table 1. *P.m.r.* chemical shifts (at 250 MHz) in p.p.m. (standard precision 0.002 p.p.m.) downfield from sodium 4,4-dimethyl-4 silapentanesulphonate and coupling constants in Hz (standard precision 0.2 Hz), at pD 7.4 in 0.1 M-phosphate buffer in $^2\text{H}_2\text{O}$

	δ_α	δ_β	$\delta_{\beta'}$	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Phe	3.983	3.276	3.116	5.2	8.05	14.7
$[\alpha\text{-}^2\text{H}]\text{Phe}$	—	3.270	3.121	—	—	14.75
Asp	3.884	2.802	2.672	3.65	8.9	17.4
$[\alpha\text{-}^2\text{H}]\text{Asp}$	—	2.604	2.499	—	—	17.5

100 μmol of L-aspartate previously neutralized with 100 μmol of NaO^2H , and about 60 units or 5 units of enzymes respectively in 1 ml of 0.1 M-potassium phosphate buffer in $^2\text{H}_2\text{O}$. The purified and freeze-dried enzyme redissolved in buffered $^2\text{H}_2\text{O}$ was added to initiate the reaction. The pH measurements (Ingold 405-M3 ultra-micro glass electrode) were made before and after addition of enzyme (pD 7.4; Glascoe & Long, 1960). The specific enzymic activity of each sample was measured immediately after the end of the kinetic measurements by the conventional test (Chesne *et al.*, 1975). At appropriate time intervals, the n.m.r. spectra were taken on a JEOL C 60 HL or on a CAMECA 250 n.m.r. spectrometer (temperature 30°C). A first-order treatment was applied with substrate concentrations measured by the area under the curves to determine the rate constant of the deuteration for different enzyme concentrations.

Analytical procedures

The deuterated amino acid was recovered, after boiling and centrifuging the reaction mixture, by chromatography on Sephadex G-25; it was then freeze-dried. The L- $[\text{}^2\text{H}]$ phenylalanine was identified by u.v. (λ_{max} 258 nm with ϵ 193 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$), by specific rotation (α_D^{20} -32.5°) on a Perkin-Elmer 141 polarimeter and by mass spectroscopy of the ethyl ester [mass measurement on the molecular peak and the fragment peaks carrying the introduced ^2H (Bieman *et al.*, 1961) with an AEI MS 30 apparatus with the following conditions: introduction temperature 300°C, ionizing current 100 μA , electron energy 70 eV].

The observed n.m.r. molecular parameters at pD 7.4, slightly different from those published at different pH values (Roberts & Jardetzky, 1970; Kainosho & Ajisaka, 1975), are given in Table 1. The non-equivalence $\delta_\beta - \delta_{\beta'}$ is slightly smaller for deuterated compounds. β and β' chemical shifts were assigned according to a small isotopic effect (Gutowsky, 1959).

Results

The partial conversion of the enzyme-bound pyridoxal phosphate after its reaction with either L-aspartate or L-phenylalanine is indicated by the appearance of a new absorption band at 335 nm

Fig. 1. Absorption spectrum of the enzyme at pH 5.4 (0.2 M-sodium acetate buffer)

1, Alone or in the presence of 5 mM-L-serine; 2, in the presence of 5 mM-L-phenylalanine; 3, in the presence of 5 mM-L-aspartate or -L-glutamate.

(Fig. 1). Similar spectral shifts are produced by both amino acids at a concentration of 5 mM, and most importantly in the acid range, since the pyridoxal form of the holoenzyme in the absence of substrate absorbs at a much higher wavelength (435 nm). Amino acids that are not substrates for this enzyme, e.g. L-serine, do not produce any change in the spectrum of the enzyme.

Fig. 2. *P.m.r. spectra (at 250 MHz) of the α - (ii) and β - (i) hydrogen atoms of sodium L-aspartate*
The reaction mixture (see the Materials and Methods section) contained 1.5 units of enzyme/ml at 30°C at times (a) 0 min, (b) 28 min, (c) 62 min, (d) 94 min and (e) 240 min.

Table 2. Initial rate of the disappearance of non-deuterated amino acid for different enzyme concentrations

Phe	Enzyme concentration (units/ml)	15.1	19.3	20	21.5	26	32.3	34.5	45.3	62.4	122
	v_0 ($\mu\text{M}\cdot\text{s}^{-1}$)	6.2	15.2	16.3	18.3	29.0	62.8	61.7	66.7	140.0	190.0
Asp	Enzyme concentration (units/ml)	0.33	0.55	0.99	1.38	1.98	10.8				
	v_0 ($\mu\text{M}\cdot\text{s}^{-1}$)	2.5	8.3	11.2	12.8	28.2	216.0				

The p.m.r. spectra of 0.1 M-sodium L-aspartate and L-phenylalanine in $^2\text{H}_2\text{O}$ are shown in Fig. 2, and allow identification of the hyperfine structures corresponding to the α -proton and to the two protons linked to the β -carbon. Addition of the pure enzyme in both cases leads to the complete disappearance of the resonance of the α -proton, and the correlative alteration of the β -peaks due to the suppression of any coupling between the α - and β -positions.

L-[2- ^2H]Phenylalanine was recovered from the reaction mixture, purified and identified (as described in the Materials and Methods section). This is the first reported synthesis of this compound by this method. D-Phenylalanine was not deuterated in $^2\text{H}_2\text{O}$ by aspartate aminotransferase, indicating, as expected, that this exchange was stereospecific.

The reaction appears to be first-order with respect to the substrate. With respect to the enzyme it is first-order for aspartate α -deuteration, and slightly greater than one for phenylalanine α -deuteration.

Deuteration of L-aspartate is at least 10 times faster than deuteration of L-phenylalanine in the presence of the same concentration of enzyme (Table 2) (e.g. the initial rate, v_0 , is $280\mu\text{M}\cdot\text{s}^{-1}$ for L-aspartate and $6.2\mu\text{M}\cdot\text{s}^{-1}$ for L-phenylalanine, for 15 units of enzyme/ml). This recalls the much greater efficiency observed with this enzyme for the aminotransferase reaction when L-aspartate is used instead of L-phenylalanine: the K_m values are 0.35 M and 80 mM respectively, and the ratio of $V_{\max}$ values is about 20:1 in favour of L-aspartate (Chesne *et al.*, 1975).

Only a slightly lower value for deuteration rate relative to transamination rate is observed. The initial rate of deuteration of 0.1 M-sodium aspartate by 1 unit of enzyme/ml is (Table 2) $11.2\mu\text{M}\cdot\text{unit}^{-1}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$ (i.e. $11.2\text{nmol}\cdot\text{unit}^{-1}\cdot\text{s}^{-1}$) and that of transamination (definition of 1 unit of enzyme) is $1\mu\text{mol}\cdot\text{unit}^{-1}\cdot\text{min}^{-1}$ (i.e. $16.6\text{nmol}\cdot\text{unit}^{-1}\cdot\text{s}^{-1}$).

The rate of α -deuterium exchange is only moderately increased in the presence of an acceptor 2-oxo acid (phenylpyruvate, oxaloacetate or 2-oxoglutarate). The experimental measurements show that this initial rate increases from 19 to $28\mu\text{M}\cdot\text{s}^{-1}$ when 2-oxoglutarate is added (up to a concentration of 5 mM) to a 60 mM solution of L-phenylalanine in potassium phosphate buffer, with 22 units of enzyme/ml. It also increases from 1.1 to $2.0\mu\text{M}\cdot\text{s}^{-1}$ when a solution

of 100 mM-L-aspartate in 0.1 M-potassium phosphate buffer with 0.1 unit of enzyme/ml is made 10 mM in oxaloacetate. A similar effect has already been observed qualitatively (Hilton *et al.*, 1956).

The possibility of β -deuteration as a side reaction was examined. It was never observed with either amino acid alone. However, β -deuteration at $10\mu\text{M}\cdot\text{s}^{-1}$ (cf. α -deuteration, $70\mu\text{M}\cdot\text{s}^{-1}$) was obtained after the addition of 10 mM-oxaloacetate to 0.1 M-aspartate with 4 units of enzyme/ml. It is now well established by Cooper (1976) that this β -deuteration occurs through transamination; the oxo acid becomes deuterated through rapid enolization (Tate *et al.*, 1964; Bruice & Bruice, 1976).

Discussion

A major feature of the *E. coli* aspartate aminotransferase when compared with the mammalian transaminases (Walter *et al.*, 1975; Cooper, 1976; Babu & Johnston, 1976; Golichowski *et al.*, 1977, and literature cited herein) is its broader specificity, extending to various aromatic amino acids (Chesne & Pelmont, 1973). This is illustrated by the spectral shift of the light-absorption of the enzyme with either L-aspartate or L-phenylalanine (Fig. 1).

Two sets of experimental data considered in conjunction throw some light on the reaction mechanism.

(i) First-order rate constants were measured for a wide range of enzyme concentrations. It was possible therefore to make a valid comparison of rates obtained with substrate concentrations ranging from 1 mM to 0.1 M, and to demonstrate that the deuteration rate and the transamination rate for aspartate were of the same order.

(ii) The deuteration occurred without any oxo acid additions, contrary to previously published procedures where oxo acid was always present (Cooper, 1976; Babu & Johnston, 1976; Golichowski *et al.*, 1977). Thus the deuteration can occur without any overall transamination and its increase after addition of an oxo acceptor is less than 2-fold.

These results suggest that the breaking of the $\text{C}_{(\alpha)}\text{-H}$ bond is the rate-limiting step of both reactions, deuteration and transamination, with this bacterial aspartate aminotransferase.

No β -deuteration was observed even with phenylalanine in the absence of oxo acid, in contrast with earlier observations dealing with mammalian alanine aminotransferase acting on alanine (Cooper, 1976; Babu & Johnston, 1976; Golichowski *et al.*, 1977).

We thank M. Henri Reutenauer for his assistance in obtaining the n.m.r. spectra at 250 MHz.

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