Interaction of Aspartate Aminotransferase with Amino Acids

By P. SCOTTO AND V. SCARDI

Laboratorio di Chimica delle Fermentazioni, Istituto di Chimica Organica dell'Università di Napoli, Naples, Italy

(Received 10 August 1964)

1. The capacity of various amino acids to convert the pyridoxal form of aspartate aminotransferase into the pyridoxamine form has been investigated. 2. Glutamate has the highest converting capacity; aspartate, α -aminopimelate, α -aminoadipate and other amino acids follow. 3. The converting capacity of the various amino acids assayed is connected with their structural features. 4. A possible role of amino acids as secondary substrates of aspartate aminotransferase is suggested.

Aspartate aminotransferase (L-aspartate-2-oxo-glutarate aminotransferase, EC 2.6.1.1) is known to exist in two forms, pyridoxal and pyridoxamine, which are interconvertible through reaction with the substrates (Jenkins &Sizer, 1960; Lis, Fasella, Turano & Vecchini, 1960). In the pyridoxal form the coenzyme (pyridoxal 5-phosphate) is firmly bound to the apoenzyme by an azomethine linkage between its 4-formyl group and an amino group of the protein (Bonavita & Scardi, 1958; Hughes, Jenkins & Fischer, 1962); in the pyridoxamine form the coenzyme (pyridoxamine 5-phosphate) is less firmly bound owing to the absence of the azomethine linkage.

In previous work on the binding of pyridoxal 5-phosphate to the aspartate aminotransferase of pig heart it was shown that only the pyridoxamine form of this enzyme can be easily resolved, i.e. dissociated into apoenzyme and coenzyme, by means of phosphate anions, which probably compete with the phosphate ester group of pyridoxamine 5-phosphate for the cationic sites of the apoenzyme (Scardi, Scotto, Iaccarino & Scarano, 1963*a, b*).

The conversion of the pyridoxal form of aspartate aminotransferase into the pyridoxamine form was obtained by interaction with L-aspartate or L-glutamate at room temperature for 15 min. Other amino acids and dicarboxylic acids were ineffective under the same conditions, so that it seemed that only these amino acids, which are the major substrates, can effect this conversion (Scardi *et al.* 1963*a, b*).

Owing to the importance of this conversion for the study of the coenzyme binding and the mode of action of the aspartate aminotransferase, further experiments were carried out with a series of amino acids and related compounds. In these experiments the extent of conversion of the pyridoxal form of the enzyme into the pyridoxamine form has been meas-

ured by means of the resolution procedure with phosphate anions, which permits one to distinguish between the two forms even if they are present in catalytic amounts (Scardi *et al.* 1963*a, b*; Bonavita, Guarneri & Scardi, 1964).

The results of these experiments are reported in the present paper. A preliminary communication on this subject was made at the First Meeting of the Federation of European Biochemical Societies, held in London on 22–25 March 1964.

MATERIALS AND METHODS

Chemicals. Pyridoxal 5-phosphate and pyridoxamine 5-phosphate were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; L-aspartic acid, L-glutamic acid, DL- α -aminopimelic acid, DL- α -aminoadipic acid, L- α -aminobutyric acid, γ -aminobutyric acid, L-histidine, L-proline, L-arginine, δ -amino-*n*-valeric acid, isopropylamine, isopentylamine and α -oxoglutaric acid were products from L. Light and Co. Ltd., Colnbrook, Bucks.; glycine, L-valine and L-lysine were from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; L-phenylalanine, glutamic acid monomethyl ester (this product needed further purification before use), glutamic acid diethyl ester and aspartic acid diethyl ester were from the Mann Research Laboratories, New York, N.Y., U.S.A.; L-alanine and L-glutamine were from Hoffmann-La Roche, Basel, Switzerland; L-tyrosine and DL- α -aminocaproic acid were from the Distillation Products Industries, Rochester, N.Y., U.S.A.; DL-leucine was from Fluka A.G., Basel, Switzerland. Aspartic acid monoethyl ester was prepared in our Laboratory. All other chemicals were pure products obtained from various sources.

Preparation and assay of the enzyme. Aspartate aminotransferase was prepared from pig heart by the method of Jenkins, Yphantis &Sizer (1959) and further purified by column electrophoresis on Sephadex G-50. The electrolyte was 0.05 M-sodium phosphate buffer, pH 7.5, and it was also used for eluting the enzyme (i.e. the faster-migrating zone) after 8 hr. run at 4°. The enzyme precipitated from the eluate with ammonium sulphate (70%, w/v) had a specific

activity of 48.5, expressed as μ moles of oxaloacetic acid formed/mg. of protein/min. at 37° under the conditions described by Cammarata & Cohen (1951). It was totally in the pyridoxal form and was proved to be homogeneous by analytical ultracentrifugation (Fig. 1).

The enzyme activity was measured in a Beckman model DB spectrophotometer equipped with a Sargent model SRL recorder. The temperature of the cell compartment was kept constant within 0.1° by a constant circulating water bath.

Protein concentration was determined spectrophotometrically as described by Kalckar (1947); in this case a Unicam SP.500 spectrophotometer was used.

Interaction of the enzyme with amino acids. A given amount of aspartate aminotransferase (about $200 \mu\text{g.}$) was incubated at 37° for 10 min. in the presence of graded concentrations of the amino acid or related compound under examination, in a final volume of 1 ml. Each compound had been dissolved in water and the pH of the solution adjusted to 7.0 unless otherwise stated. The extent of interaction leading to the conversion of the pyridoxal form of the enzyme into the pyridoxamine form was calculated in terms of resolution; this was performed by adding 1 ml. of $\text{M-KH}_2\text{PO}_4$ to the incubation mixture and reincubating for 15 min. more at the same temperature. Under these conditions only the pyridoxamine form of the enzyme is resolved. Then portions were taken and assayed for enzyme activity with and without the addition of an excess of pyridoxal 5-phosphate. The degree of resolution was calculated as reported by Scardi *et al.* (1963a,b), i.e. percentage resolution = $100 (A - a)/A$, where A and a are the enzyme activities measured in the presence and in the absence of pyridoxal 5-phosphate respectively. In general A was equal or almost equal to the activity of the enzyme before resolution, thus showing that no denaturation of the apoenzyme occurred.

RESULTS AND DISCUSSION

Interaction with amino acids. The interaction between the pyridoxal form of aspartate amino-

transferase and certain amino acids causes a conversion into the pyridoxamine form, the extent of which can be measured in terms of percentage resolution after the action of the phosphate anions. Fig. 2 shows the percentage conversion of one form of the enzyme into the other as a function of the logarithm of the reciprocal molar concentration of several amino acids. Where racemates are used, the concentration given in the Figure refers to the L-form only, since it was shown by Scardi *et al.* (1963a,b) that only L-amino acids interact with the enzyme. Glutamate has the highest affinity, followed by aspartate, as was to be expected on the basis of the data of Velick & Vavra (1962). This accounts for our previous findings in which both glutamate and aspartate, being the major substrates of aspartate aminotransferase, seemed to be the only amino acids capable of converting the pyridoxal form of the enzyme into the pyridoxamine form. The sigmoid curves are almost parallel to each other.

To compare the affinities of different compounds for the pyridoxal form of the enzyme, the logarithm of the reciprocal concentration giving a 50% conversion was taken as a reference value and indicated as 'converting capacity' or pC_{50} . In Table 1 several other amino acids of different type are compared in their capacity to convert the pyridoxal form of aspartate aminotransferase into the pyridoxamine form. The monoamino-dicarboxylic acids show the highest converting capacity, though they do not follow a well-defined rule, such as the distance between the two carboxyl groups. It seems, however, that the converting capacity alternates according to the number of carbon atoms: amino acids having an odd number of carbon atoms are more effective than their higher or lower homologues having an even number of carbon atoms. This alternation is

Fig. 1. Sedimentation patterns of the pig-heart aspartate aminotransferase preparation used in our experiments. Schlieren diagrams were photographed at 8 min. intervals in a Spinco model E analytical ultracentrifuge. Other conditions were: 50740 rev./min., temperature 20.8° and protein concentration 0.82 mg./ml. of 0.1 M-potassium phosphate buffer, pH 7.4.

Fig. 2. Conversion of the pyridoxal form of aspartate aminotransferase into the pyridoxamine form by interaction with several amino acids. The extent of conversion is measured by the resolution method described in the text. $\circ$, Glutamate; $\bullet$, aspartate; Δ , α -aminopimelate; $\bullet$, α -aminoadipate; $\square$, α -aminobutyrate; $\blacktriangle$, alanine.

Table 1. Capacity of different amino acids to convert the pyridoxal form of aspartate aminotransferase into the pyridoxamine form

The converting capacity (pC_{50}) is expressed as the logarithm of the reciprocal molar concentration of the amino acid (L-form) giving a 50% conversion of the enzyme under the experimental conditions described in the Materials and Methods section. —, Not determined.

Amino acid	Formula	Converting capacity (pC_{50})		
		pH 5.5	pH 7.0	pH 8.5
Aspartic acid	$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot CO_2H$	5.2	5.5	5.75
Glutamic acid	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_2 \cdot CO_2H$	6.2	6.5	6.70
α -Aminoadipic acid	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_3 \cdot CO_2H$	—	3.4	—
α -Aminopimelic acid	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_4 \cdot CO_2H$	3.7	4.1	4.40
Glycine	$HO_2C \cdot CH(NH_2) \cdot H$	—	None	—
Alanine	$HO_2C \cdot CH(NH_2) \cdot CH_3$	2.3	2.65	2.90
α -Aminobutyric acid	$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot CH_3$	1.8	3.00	3.30
α -Aminocaproic acid	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_5 \cdot CH_3$	—	1.10	—
Valine	$HO_2C \cdot CH(NH_2) \cdot CH(CH_3)_2$	—	None	—
Leucine	$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot CH(CH_3)_2$	—	3.05	—
Phenylalanine	$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot C_6H_5$	2.0	3.25	3.90
Histidine	$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot C_3H_3N_2$	—	2.15	—
Lysine	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_3 \cdot CH_2 \cdot NH_2$	—	1.30	—
Arginine	$HO_2C \cdot CH(NH_2) \cdot [CH_2]_2 \cdot CH_2 \cdot NH \cdot C(NH_2) : NH$	—	2.00	—
γ -Aminobutyric acid	$HO_2C \cdot [CH_2]_3 \cdot NH_2$	—	2.30	—
δ -Amino- <i>n</i> -valeric acid	$HO_2C \cdot [CH_2]_4 \cdot NH_2$	—	0.70	—

not unique in chemistry (e.g. the melting points and solubilities of dicarboxylic acids follow the same rule).

Monoamino-monocarboxylic acids show a definite though smaller converting capacity, which seems to increase with the length of the carbon chain, reaching a maximum at a number of carbon atoms that we could not evaluate exactly, after which it decreases. Valine has no converting capacity, whereas leucine has a converting capacity as high as that of α -aminobutyric acid; this can be explained by comparing the lengths of the non-polar portion of their molecules. As far as aromatic amino acids are concerned, phenylalanine has a converting capacity almost equal to that of the less effective monoamino-dicarboxylic acid. The converting capacity of tyrosine could not be measured because of its very low solubility at the pH values of our experiments. Histidine follows in order of decreasing capacity. Proline is ineffective, but two di-amino-monocarboxylic acids, lysine and arginine, have pC_{50} values of 1.3 and 2.0 respectively.

The position of the amino group seems to be important, as shown by the results obtained with γ -aminobutyric acid and δ -amino-*n*-valeric acid.

For some amino acids the pC_{50} values were also measured at pH 5.5 and 8.5 (Table 1). As a general rule, pC_{50} values increase slightly from acidic to basic pH. Only α -aminobutyric acid and phenylalanine almost double their pC_{50} values in passing from pH 5.5 to pH 8.5.

In conclusion it appears from Table 1 that several amino acids, besides glutamate and aspartate, show

Fig. 3. Effect of chemical modification of the glutamic acid molecule on its capacity of converting the pyridoxal form of aspartate aminotransferase into the pyridoxamine form. Conditions were the same as in Fig. 2. ○, Glutamate; ●, glutamate diethyl ester; △, glutamate monomethyl ester.

a higher affinity than alanine, which was reported to interact with the pyridoxal form of aspartate aminotransferase to yield pyruvate with the concurrent conversion of the enzyme into its pyridoxamine form (Lis *et al.* 1960; Jenkins, 1961). As the interaction between the pyridoxal form of aspartate aminotransferase and an amino acid represents a half reaction of enzymic transamination, the question is raised whether these amino acids, in the presence of the proper keto acid, i.e. α -oxoglutarate, behave as substrates of that enzyme. Preliminary experiments with α -aminoadipic acid and α -amino-

pimelic acid seem to indicate that this is the case (Scardi & Marino, 1964).

Interaction with amino acid derivatives. Fig. 3 shows the effect of esterification of carboxyl groups on the converting capacity of glutamic acid. Glutamate monomethyl ester is less than one-thousandth as active as glutamate, thus resembling α -amino-adipate; this is very probably due to the decreased polarity of the portion of the molecule, which ends with the esterified carboxyl group. Glutamate diethyl ester and glutamine show sigmoid curves of different slope as compared with the curves exhibited by amino acids. This is probably due to the fact that glutamate diethyl ester and glutamine resemble amines. Some simple amines were tested and only some isomeric amines proved effective: isopropylamine and isopentylamine showed sigmoid curves parallel to those exhibited by glutamate diethyl ester and glutamine and pC_{50} values of the same order of magnitude, i.e. 3.95 and 3.30 respectively. Aspartate monoethyl and diethyl esters have no effect at all on the pyridoxal form of aspartate aminotransferase.

Our experimental results, indicating that conversion of the pyridoxal form of aspartate aminotransferase into the pyridoxamine form can be performed by any amino acid having certain structural features, should contribute to the understanding of the mechanism of action of this enzyme. Further, the results contribute towards the present know-

ledge of the number of possible substrates of aspartate aminotransferase.

The present research was made possible through the support and the sponsorship of the U.S. Army Department, through its European Research Office.

REFERENCES

- Bonavita, V., Guarneri, R. & Scardi, V. (1964). *Life Sci.* **3**, 889.
Bonavita, V. & Scardi, V. (1958). *Experientia*, **14**, 7.
Cammarata, P. S. & Cohen, P. P. (1951). *J. biol. Chem.* **193**, 45.
Hughes, R. C., Jenkins, W. T. & Fischer, E. H. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1615.
Jenkins, W. T. (1961). *J. biol. Chem.* **236**, 474.
Jenkins, W. T. & Sizer, I. W. (1960). *J. biol. Chem.* **235**, 620.
Jenkins, W. T., Yphantis, D. A. & Sizer, I. W. (1959). *J. biol. Chem.* **234**, 51.
Kalckar, H. (1947). *J. biol. Chem.* **167**, 461.
Lis, H., Fasella, P., Turano, C. & Vecchini, P. (1960). *Biochim. biophys. Acta*, **45**, 529.
Scardi, V. & Marino, G. (1964). *Boll. Soc. ital. Biol. sper.* **40**, 723.
Scardi, V., Scotto, P., Iaccarino, M. & Scarano, E. (1963a). In *Chemical and Biological Aspects of Pyridoxal Catalysis*, p. 167. Ed. by Snell, E. E., Fasella, P., Braunstein, A. E. & Rossi Fanelli, A. Oxford: Pergamon Press Ltd.
Scardi, V., Scotto, P., Iaccarino, M. & Scarano, E. (1963b). *Biochem. J.* **88**, 172.
Velick, S. F. & Vavra, J. (1962). *J. biol. Chem.* **237**, 2109.