J. Enzyme Inhibition, 2001, Vol. 16, pp. 251–257
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Effects of Substrate Structural Analogues on the Enzymatic Activities of Aspartate Aminotransferase Isoenzymes

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(Received 23 August 2000)

Aspartate aminotransferase (AAT, EC 2.6.1.1) catalyses the transamination of L-aspartate to oxaloacetate. It has been reported that AAT from different plant sources can catalyse the transamination of other compounds structurally similar to the natural substrates.

Specificity and kinetic studies were performed with two aspartate aminotransferase isoenzymes (AAT-1 and AAT-2) from leaves of *Lupinus albus* L. cv Estoril using different amino donors and acceptors. Both isoenzymes showed residual activity for some of the substrates tested. Competitive inhibition was found with most of the structural analogues which is typical of a *ping-pong bi-bi* kinetic mechanism.

It was found that both isoenzymes can use 2-amino-4-methoxy-4-oxobutanoic acid as amino donor. AAT-2 uses 2-amino-4-methoxy-4-oxobutanoic acid at a similar rate as L-aspartate but AAT-1 uses this substrate at a slower rate. The use of this amino donor by AAT isoenzymes has not been reported previously, and our results indicate structural differences between both isoenzymes.

Keywords: Aspartate aminotransferase, Isoenzymes, Kinetic, Inhibitors, Substrate specificity

Abbreviations: AAT, aspartate aminotransferase; GS/GOGAT, glutamine synthetase/glutamate synthase; AMOB, L-2-amino-4-methoxy-4-oxobutanoic acid; AMOP, L-2-amino-5-methoxy-5-oxopentanoic acid

INTRODUCTION

Aspartate aminotransferase (AAT, EC 2.6.1.1) is a pyridoxal 5′-phosphate dependent enzyme that catalyses the reversible transfer of an amino group from aspartate to 2-oxoglutarate to form oxaloacetate and glutamate. In plants AAT plays an important role in numerous metabolic processes, namely protein synthesis, ammonia assimilation in the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, carbon shuttle in C_4 plants and in the malate-aspartate hydrogen shuttle across membranes.[1]

Plants contain multiple AAT isoenzymes which are associated with different organelles, and these have been studied in several plant species.[2,3,4,5]

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It is known that AAT from different plant sources can catalyse the transamination of some other compounds structurally similar to the natural substrates. Turano *et al.*[5] and Reynolds *et al.*[6] did not detect any AAT activity with other substrates for two isoenzymes from lupine root nodules. However, other authors have shown that some residual activity can be detected using other amino donors and amino acceptors from various plant sources.[7,8]

Some earlier substrate specificity studies were also carried out with different substances.[9,10,11] However, some of the reported activities may have been caused by the presence of other enzymes due to a low degree of purification.

Moreover, inhibition studies of substrate analogues have not been reported in plants. However, such knowledge is important in gaining more complete information about how these substrate analogues affect AAT activity, and helps to clarify the way these substances affect enzyme activity.

We present the results of studies on the substrate specificity of two AAT isoenzymes (designated AAT-1 and AAT-2) from the leaves of white lupine (*Lupinus albus* L. cv Estoril). Several amino acids and structural analogues of both amino donors and acceptors were investigated. Some of the compounds used have not been reported in previous studies on substrate specificity. The kinetic constants and inhibition pattern of the isoenzymes using some of the structural analogues were also determined. The kinetics of the reaction of transamination with L-2-amino-4-methoxy-4-oxobutanoic acid (AMOB) was also studied.

MATERIAL AND METHODS

Plant Material

Leaves of white lupine plants (*Lupinus albus* L. cv Estoril) were collected after 4 weeks growth in nutrient solution, under controlled environment conditions of 18 °C and 55% humidity, with 14/10 h dark/light periods.

Enzyme Extraction

Leaves (7 g) were macerated in 10 mL of 10 mM Tris-HCl buffer (pH 8.0), containing 0.2 mM pyridoxal 5′-phosphate (PLP) and 5 mM 2-mercaptoethanol, in the presence of 2% (w/w) insoluble polyvinylpolypyrollidone (PVP). The homogenate was centrifuged for 30 min at 20 000 X*g* and filtered through 0.2 μm membranes. All procedures were carried out at a temperature below 4 °C. The enzyme extract obtained was subjected to a temperature fractionation (4 min at 45 °C) and salt fractionation with ammonium sulphate (25–80%) followed by centrifugation (20 000 X*g* for 15 min) and filtration through 0.2 μm membranes.

Separation and Purification of AAT-1 And AAT-2

The volume of the enzyme extract was reduced to 7 mL by ultra-filtration using a Diaflo concentrator (with YM10 *Millipore* membranes), and loaded on a gel exclusion chromatography column (Sephacryl S-200 HR, *Pharmacia*, 86 × 2.6 cm) equilibrated in 20 mM *Tris*-HCl (pH 8.0) containing 2 mM 2-mercaptoethanol. The peak fractions containing AAT activity were pooled, desalinised and the volume reduced to 8 mL and applied to an anion exchange chromatography column (Mono-Q 5/5, *Pharmacia*) equilibrated in 20 mM *Tris*-HCl (pH 8.0) containing 2 mM 2-mercaptoethanol. AAT isoenzymes were eluted using a linear salt gradient, 100 to 300 mM NaCl in 20 mM *Tris*-HCl (pH 8.0) containing 2 mM 2-mercaptoethanol. AAT-1 and AAT-2 were separately eluted at 200 and 150 mM NaCl, respectively. Fractions containing either isoenzyme were pooled and desalinised with an Amicon membrane (microcon-10). The separation and purification of AAT isoenzymes was confirmed by SDS-PAGE.[12]

During the purification process, protein content was evaluated by measuring the absorbance at 280 nm.

Enzyme Assay

AAT activity in the reverse reaction, was measured by the decrease in absorbance at 260 nm as oxaloacetate was consumed[13] and varying the amino donor at a fixed concentration of oxaloacetate. One International Unit is defined as the amount of enzyme that catalyses the utilisation of 1 μmol of oxaloacetate per min, at 25 °C ($\epsilon = 1.2 \times 10^2\,L\,mol^{-1}\,cm^{-1}$). AAT activity was also assayed by the coupled glutamate dehydrogenase method[5] varying the amino acceptor at a fixed concentration of L-glutamate. One International Unit is defined as the amount of enzyme that catalyses the oxidation of 1 μmol NADH per min, at 25 °C ($\epsilon = 6.3 \times 10^2\,L\,mol^{-1}\,mm^{-1}$). In the forward reaction, AAT activity was assayed by monitoring the disappearance of NADH spectrophotometrically at 340 nm in the coupled malate dehydrogenase reaction[14] and varying the amino acceptor at a fixed concentration of L-aspartate. One International Unit is defined as the amount of enzyme that catalyses the oxidation of 1 μmol NADH per min, at 25 °C. This method was also used to measure the AAT activity with AMOB as alternative substrate to L-aspartate.

AAT activity was also assayed by the coupled glutamate dehydrogenase method, by varying the amino donor at a fixed concentration of 2-oxoglutarate. One International Unit is defined as the amount of enzyme that catalyses the reduction of 1 μmol NAD^+ per min, at 25 °C.

Soluble protein was determined by the Lowry method[15] with bovine serum albumin as the standard.

Kinetic Studies

The kinetic parameters K_m and V_{max} for both isoenzymes and the apparent inhibition constants were calculated using the direct linear plot methodology applied to the *ping-pong bi-bi* kinetic mechanism.[16] In the inhibition studies the inhibition pattern was deduced from Lineweaver-Burk plots.[17]

RESULTS AND DISCUSSION

Substrate Specificity

Substrate specificity studies were performed for the two separated AAT isoenzymes in both the forward and reverse reaction, using several different amino donors and amino acceptors (Table I). As expected, both isoenzymes showed high specificity towards the amino acceptors oxaloacetate and 2-oxoglutarate, when the amino donor was L-glutamate and L-aspartate, respectively. Residual activity values were detected for some of the alternative substrates used. This means that they can undergo a transformation by both AAT isoenzymes.

Low activity levels were detected with L-asparagine and L-glutamine (in the forward reaction only), with L-glycine (in the reverse reaction) and with L-alanine (in the reverse reaction, but only for AAT-1).

Yagi *et al.*[8] also detected residual activities for AAT isoenzymes, extracted from rice bran, with some of the amino donor and acceptors studied. They showed that with L-cysteine sulfinate as amino donor, AAT had high activity levels. Residual activity levels were found with L-phenylalanine, L-leucine and L-valine as amino donors and with *p*-hydroxyphenylpyruvate, phenylpyruvate and pyruvate as amino acceptors.

Effect of AMOB

Both isoenzymes showed high enzymatic activity with AMOB as amino donor substrate. This meant that AMOB not only had a greater affinity for the enzyme binding-site than the other analogues studied but also that it could be an

TABLE I Substrate specificity of AAT-1 and AAT-2 isoenzymes

Amino acceptor (2 and 0.2 mM)	Relative activity (%)*			
	AAT-1		AAT-2	
	L-Aspartate (20 mM)	L-Glutamate (20 mM)	L-Aspartate (20 mM)	L-Glutamate (20 mM)
Oxaloacetate	–	100.0	–	100.0
2-Oxoglutarate	100.0	–	100.0	–
Hydroxypyruvic acid	0.0	0.0	0.0	0.0
Glyoxylic acid	0.0	1.6	0.0	0.0
Pyruvic acid	0.0	0.1	0.0	0.3
Succinic acid	0.2	0.0	0.2	0.0
Fumaric acid	0.0	0.0	0.0	0.0
Maleic acid	0.0	0.3	0.0	0.0
Glutaric acid	0.0	0.0	0.0	0.0
L-Cysteic acid	0.0	0.0	0.0	0.0
Amino donor (20 mM)	**2-Oxoglutarate (2 mM)**	**Oxaloacetate (0.2 mM)**	**2-Oxoglutarate (2 mM)**	**Oxaloacetate (0.2 mM)**
L-Aspartate	100.0	–	100.0	–
L-Glutamate	–	100.0	–	100.0
L-Asparagine	3.5	0.0	5.1	0.0
L-Glutamine	2.5	0.0	5.4	0.0
L-Alanine	0.9	2.0	0.0	0.0
L-Glycine	0.0	8.6	0.0	1.0
L-Proline	0.0	0.0	0.0	0.0
L-Arginine	0.0	0.0	0.0	0.0
L-Lisyne	0.0	0.0	0.0	0.0
AMOB	63.5	2.8	84.3	4.2
AMOP	0.0	0.0	0.0	0.0

*Mean of 3 determinations.

alternative amino donor to L-aspartate. Kinetic studies were performed by measuring the isoenzyme activity at fixed concentrations of AMOB (between 3 and 20 mM), and with varying concentrations of the other substrate, 2-oxoglutarate (Figure 1). The kinetic mechanism of transamination with AMOB as amino donor was also of the *ping-pong bi-bi* type for both isoenzymes since the primary plots obtained exhibited a parallel line pattern. The possibility of any interference by AMOB in the coupled assay conditions was ruled out by the use of a different enzyme activity method (coupled glutamate dehydrogenase method in forward direction). Based on the structure of AMOB it is predicted that the product of the transamination will be 4-methoxy-2,4-dioxobutanoic acid (structurally similar to oxaloacetate).

As was suggested from the use of L-cysteic acid, it is likely that the carboxylic group bonded to C_α is a major determinate of the binding of the substrate to the enzyme.

The kinetic constants obtained with AMOB as amino donor substrate and 2-oxoglutarate as amino acceptor are presented in Table II. The K_m values suggest that AAT-2 has a greater affinity for AMOB than AAT-1. These values can be the result of the differences between the structures of the two isoenzymes at the primary structure level, which are characteristic of isoenzymes. A comparison of the kinetic constants for AMOB with those obtained for L-aspartate, under identical experimental conditions ($K_m = 2.26$ mM and $V_{max} = 2.97\ U\ mg^{-1}$ for AAT-1 and $K_m = 3.33$ mM and $V_{max} = 0.15\ U\ mg^{-1}$ for AAT-2)[12] showed that AAT-2 uses AMOB at a

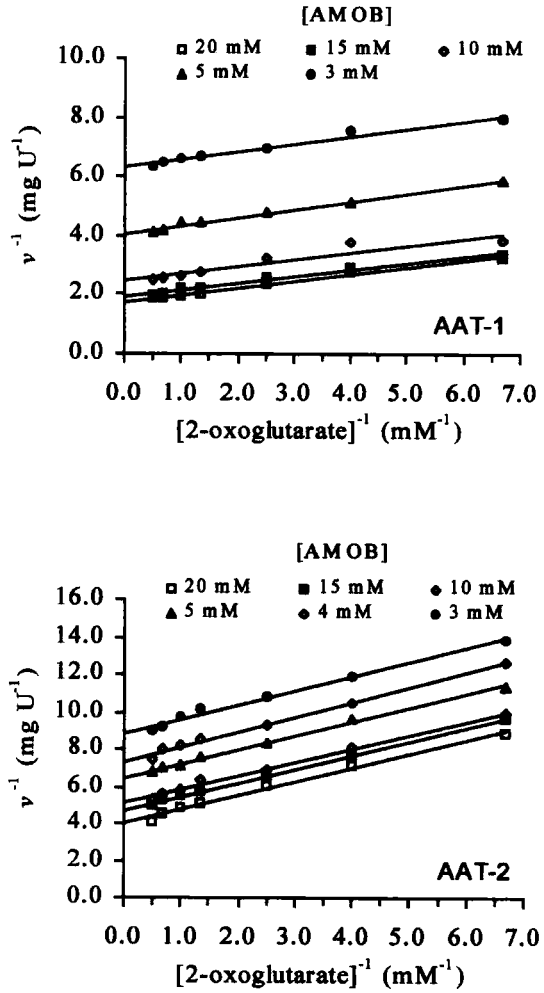

FIGURE 1 Primary plots obtained with AMOB as amino donor substrate and 2-oxoglutarate as amino acceptor (for AAT-1 and AAT-2 isoenzymes).

TABLE II Kinetic parameters for AAT-1 and AAT-2 isoenzymes, obtained with AMOB as amino donor substrate and 2-oxoglutarate as amino acceptor

	K_m^{AMOB} (mM)	V_{max}^{AMOB} (U mg^{-1})
AAT-1	20.31 ± 2.20	1.26 ± 0.12
AAT-2	4.09 ± 0.33	0.28 ± 0.01

rate similar to that for L-aspartate. Although AAT-1 can also use AMOB as an amino donor, it does so at a much slower rate. The use of AMOB as an amino donor substrate of transamination by AAT has not been previously reported.

Inhibition Studies

A number of the structural analogues used here in the substrate-specificity studies were tested for their ability to inhibit AAT activity (Table III). The corresponding apparent kinetic constants of inhibition, K_i, were determined (Table IV). A competitive-type inhibition was found for five of the analogues studied. This inhibition pattern was in agreement with a *ping-pong bi-bi* kinetic mechanism, characteristic of aminotransferases.[17] Competitive inhibition behaviour is indicative of the affinity of the inhibitor for the enzyme binding-site and the different degree of inhibition can be explained by the degree of structural similarity between the substrate analogues. Kiick and Cook,[18] with pig-heart AAT, referred to the stereospecificity and the distance between carboxylic groups as important structural factors that affect the inhibition behaviour.

Maleic and fumaric acid (*cis* and *trans* butendioic acid respectively) showed very different effects on the AAT isoenzymes activity. Although competitive-type inhibition was observed, maleic acid caused a much greater inhibition (lower K_i values) than fumaric acid. This is probably due to the fact that the double bond between C_α and C_β did not allow the rotation necessary to achieve a more favourable position for a better approach of fumaric acid to the binding site. Christen and Metzler[19] came to a similar conclusion for mammalian AAT isoenzymes. This difference was smaller for AAT-2 than for AAT-1, showing that the influence of the stereochemical orientation is more important for AAT-1 although the carboxylic groups of fumaric acid (*trans*-butendioic acid) were not in a favourable position to be attached to the binding site, in both isoenzymes.

The distance between carboxylic groups could explain the greater inhibition effect of succinic acid (C_4) and glutaric acid (C_5) with respect to 2-oxoglutarate (lower K_i values). K_i values for L-cysteic acid seems to indicate that the α-carboxylic group is crucial for the affinity of the

TABLE III Inhibition pattern obtained with structural analogues at a constant and non-saturating concentration of L-aspartate and 2-oxoglutarate

	AAT-1		AAT-2	
	L-Aspartate (2 mM)	2-Oxoglutarate (0.2 mM)	L-Aspartate (2 mM)	2-Oxoglutarate (0.2 mM)
Hydroxypyruvic acid	NI*	NI	NI	NI
Glyoxylic acid	NI	NI	NI	NI
Pyruvic acid	NI	NI	NI	NI
Succinic acid	C*	C	C	C
Fumaric acid	C	C	C	C
Maleic acid	C	C	C	C
Glutaric acid	C	C	NI	NI
L-Cysteic acid	C	C	C	C
AMOP	NI	NI	C	NI

*NI – No inhibition; C – Competitive inhibition.

TABLE IV Inhibition constants obtained with structural analogues at a constant and non-saturating concentration of L-aspartate and 2-oxoglutarate

Structural analogues	AAT-1		AAT-2	
	K_i with respect to 2-oxoglutarate (mM)	K_i with respect to L-aspartate (mM)	K_i with respect to 2-oxoglutarate (mM)	K_i with respect to L-aspartate (mM)
Succinic acid	10.66 ± 1.75	36.89 ± 1.11	8.86 ± 0.76	16.84 ± 0.29
Fumaric acid	24.59 ± 1.25	44.12 ± 11.12	6.78 ± 0.38	33.72 ± 5.16
Maleic acid	1.40 ± 0.04	10.58 ± 0.35	1.21 ± 0.01	8.48 ± 0.06
Glutaric acid	15.05 ± 0.15	26.55 ± 8.36	–	–
L-Cysteic acid	16.29 ± 1.45	97.63 ± 13.67	20.73 ± 2.86	150.79 ± 36.49
AMOP	–	–	25.85 ± 0.93	–

analogue to the binding-site. Differences in the structures of the two isoenzymes can justify the different inhibition behaviour observed with glutaric acid and L-2-amino-5-methoxy-5-oxo-pentanoic acid (AMOP).

CONCLUSIONS

Both aspartate aminotransferase isoenzymes showed residual activity values with some of the alternative substrates used and higher activity values were obtained with AMOB (structurally similar to L-aspartate).

A competitive-type inhibition was found for some of the structural analogues studied, in agreement with the *ping-pong bi-bi* kinetic mechanism of aminotransferases. This was related to a structural similarity between the substrate analogues that determines a different affinity of the inhibitor molecule for the enzyme binding-site.

Competitive-type inhibition was observed with all structural analogues studied. The different degree of inhibition can be explained by the existence of a double bond in *cis* (maleic acid) and *trans* (fumaric acid) butendioic acid. The distance between carboxylic groups can also explain the greater inhibition effect of succinic acid (C_4) and glutaric acid (C_5) with respect to 2-oxoglutarate (lower K_i values). Moreover, it appears that the carboxylic group bounded to C_α is a strong determinant of the binding of the substrate to the enzyme.

For AAT-1 and AAT-2 some differences were found in the affinity of the inhibitors to the

binding site and this can be a consequence of the structural differences in the amino acid sequence, characteristic of isoenzymes. AMOB can be used as an amino donor substrate by both isoenzymes, following a *ping-pong bi-bi* kinetic mechanism. The K_m values suggest that AAT-2 had a greater affinity for AMOB than AAT-1. AAT-2 uses AMOB at a rate similar to that for L-aspartate although AAT-1 can also use AMOB as an amino donor but at a much slower rate.

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