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INFLUENCE OF RING- AND SIDE-CHAIN SUBSTITUENTS ON THE TRANSAMINATION OF AROMATIC AMINO ACIDS BY TWO MULTISPECIFIC ASPARTATE-AROMATIC AMINOTRANSFERASE ISOZYMES PURIFIED FROM BUSHBEAN SHOOTS*

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Key Word Index—*Phaseolus vulgaris*; *Leguminosae*; bushbean; aminotransferases; multispecific; aromatic amino acids; analogues; mechanism of activation.

Abstract—The breadth of substrate specificity shown by the multispecific aspartate-aromatic aminotransferase of bushbean (*Phaseolus vulgaris*) has been investigated by testing the ability of two cytosolic isozymes (I and II), purified from shoot tissue, to catalyse transamination reactions between a range of ring- and sidechain-substituted aromatic amino acids and 2-oxoglutarate. Ring-substituted phenylalanines were the most reactive substrates whereas ring-substitution in tyrosine or tryptophan resulted in transamination rates lower than those observed with the parent amino acids. All side chain-substituted analogues were found to be totally inactive. The highest activity shown by any ring-substituted phenylalanine was observed with the 4-amino- compound, followed closely by the 4-hydroxy- and 4-halogen-compounds. In contrast, 4-nitrophenylalanine was completely inactive. These trends were consistent for both isozymes I and II, but only isozyme II showed greatly enhanced activity over that found with the parent amino acid when certain ring-substituted analogues were tested. The varying capacity of the bushbean isozymes to utilize the present range of substituted amino acids is compared with previous reports on the substrate specificity shown by aspartate and aromatic aminotransferases isolated from mammalian and microbial systems. A model for the mechanism of activation observed with bushbean isozyme II in the presence of certain 4-substituted aromatic amino acids is proposed, based on current understanding of the nature of the active site of animal aspartate aminotransferases.

INTRODUCTION

A multispecific aspartate-aromatic aminotransferase [1], purified from roots of bushbean seedlings, was found to readily catalyse the transamination reaction between L-aspartate and 2-oxoglutarate (2-OG) and also exhibited moderate activity toward the three aromatic amino acids when 2-OG was supplied. In the same year, it was reported that aspartate aminotransferases in the cytosol and mitochondrial fractions from pig heart could also utilize the three aromatic amino acids as substrates [2] and this was confirmed in a detailed study of the two enzymes [3]. In a later study [4], an aspartate-aromatic aminotransferase was isolated from extracts of *Escherichia coli* and the pure enzyme was able to catalyse the transamination of L-aspartate and each of the three aromatic amino acids when 2-OG was supplied as the amino-group acceptor. Thus, it would appear that multispecific aspartate-aromatic aminotransferases are widely distributed in living systems [5, 6].

We have recently reported the purification of two cytosolic isozymes (I and II) of aspartate-aromatic aminotransferase from bushbean shoots [7, 8] which showed similar multispecificity. These isozymes were also found to catalyse the transamination of ring-chlorinated phenylalanines to varying degrees [8]; isozyme II, for example, showed very high activity when 4-chlorophenylalanine was the substrate but much lower activity with the 2-chloro- or 3-chloro-analogues. Since it was earlier found that the aspartate-aromatic aminotransferase from bushbean roots exhibited high activity with 3-iodo-L-tyrosine [1] and that mammalian aromatic aminotransferases were reported to show activity toward several ring-substituted aromatic amino acids [9–11], we decided to investigate further the range in substrate specificity shown by the cytosolic isozymes I and II from bushbean shoots. The ability of these isozymes to catalyse transamination reactions between 2-OG and a series of side chain- and ring-substituted aromatic amino acids was examined in the present study. The capacity of the bushbean isozymes to utilize the ring-substituted aromatic amino acids is compared with earlier results reported for the aromatic aminotransferases in mammalian and microbial systems, and the data is examined in the light of current understanding of the mechanism of aspartate aminotransferase activity and the nature of its active site.

*Part 3 in the series: Metabolism of chloro-phenylalanines by multispecific aspartate-aromatic aminotransferases in crop and weed plants. This series is dedicated to the memory of our former colleague, Budhi Singh Rauthan, who first demonstrated in 1972 the *in vivo* conversion of L-phenylalanine to phenylacetic acid in higher plants. Mr Rauthan was killed in the crash of the Air India Boeing 747 into the Atlantic Ocean, 23 June 1985.

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RESULTS AND DISCUSSION

Isolation of bushbean aminotransferase isozymes I *and* II

The results obtained during purification of the aspartate-aromatic aminotransferase isozymes I and II from an enriched cytosolic fraction from 10-day-old bushbean shoots are summarized in Table 1. The procedure used to prepare a cytosolic fraction involved gentle homogenization of shoot tissue in isotonic medium followed by differential centrifugation of the homogenate to separate intact nuclei, chloroplasts, mitochondria and microbodies from the cytosol. The L-phenylalanine aminotransferase activity of this fraction was 0.300 mU/mg protein, which is considerably higher than that reported in our earlier paper [7] for a total soluble extract of an equivalent sample of bushbean shoots isolated in hypotonic medium (0.20 mU/mg protein; total protein = 3000 mg). Since the total activity in each case was *ca* 0.60 units, this would suggest that while the present enriched cytoplasmic fraction contained only 65% of the protein present in the total soluble extract [7], it contained most of the L-phenylalanine aminotransferase activity (*ca* 96%). This implies that about 35% of the total cell protein, but only 3–4% of the L-phenylalanine aminotransferase activity, was contained in the total particulate fraction. Thus, in bushbean shoots, most of the L-phenylalanine aminotransferase activity is located in the cytosol.

The enriched cytosolic fraction was then subjected to ammonium sulphate fractionation and the protein precipitated at the 40–70% saturation step was redissolved and filtered through a column of Sephacryl S-300. At this point, the L-phenylalanine aminotransferase activity had been increased about six-fold per mg protein. When the fraction exhibiting this activity was chromatographed on a DEAE-Sephacel column at pH 6.8 using a 0–250 mM KCl gradient, the L-phenylalanine aminotransferase activity was resolved into two fractions, provisionally called isozymes I and II. Each isozymic fraction was further purified by chromatography on hydroxylapatite at pH 7.5, and isozyme II was subjected to a final chromatography step on a DEAE-Sephacel column at pH 8.10. The overall purification achieved was about 280-fold with a 69% recovery of total L-phenylalanine aminotransferase activity (Table 1). Both purified isozymic fractions were shown to be free of other L-aromatic aminotransferases by PAGE followed by assay of the gel slices, as previously described [7].

The substrate specificity of isozymes I and II for each of the four common aliphatic keto acids was tested in the presence of D,L-phenylalanine and each of the monochloro-D,L-phenylalanines, with the presumption that other mono-ring-substituted aromatic amino acids would display similar keto acid specificities. Isozyme II clearly preferred 2-oxoglutarate (2-OG) as the keto acid when D,L-phenylalanine or any monochlorophenylalanine was the amino donor (Table 2). Isozyme I was able to utilize 2-OG or oxaloacetate (OAA) about equally with D,L-phenylalanine, but only 2-OG was able to support the activity of this isozyme in the presence of 3-chloro- and 4-chloro-phenylalanines. 2-Chlorophenylalanine was not metabolized by isozyme I. Neither isozyme was able to utilize glyoxylate or pyruvate as keto acid substrates with any of the amino donors supplied. Since both isozymes clearly favoured 2-OG in the presence of phenylalanine and the monochlorophenylalanines, it was chosen as the keto acid for all subsequent amino acid substrate specificity studies described below.

L-Amino acid substrate specificity and its relation to current theory on the mechanism of aminotransferase action

The substrate specificities of isozymes I and II were next tested with a group of L-amino acids containing represen-

Table 1. Purification of the aspartate-aromatic aminotransferase isozymes I and II from an enriched cytoplasmic fraction prepared from 10-day old bushbean shoots

Purification step	Protein (mg)	Total activity (units)	Sp. activity (units/mg protein $\times 10^{-3}$)*	Recovery (%)	Purification (Fold)
1. Isotonic extraction	1950	0.58	0.300	100	—
2. 40–70% $(NH_4)_2SO_4$ precipitation	1260	0.56	0.444	97	1.5
3. S-300 gel filtration	317	0.55	1.74	95	5.8
4. DEAE-Sephacel chromatography at pH 6.8: Isozyme I	63	0.15			
Isozyme II	100	0.36			
	163	0.51	3.13	87	10.5
5. Hydroxylapatite chromatography: Isozyme I	1.6	0.10			
6. Hydroxylapatite chromatography: Isozyme II	8.0	0.34			
	9.6	0.44	45.8	76	154
7. DEAE-Sephacel chromatography at pH 8.1: Isozyme II	3.2	0.30			
Plus step (5)	1.6	0.10			
	4.8	0.40	83.3	69	280

*Unit of Enzyme Activity will convert 1 μmol L-phenylalanine to 1 μmol phenylpyruvic acid per min at 35°, pH 8.5

Table 2. Keto acid substrate specificity of bushbean aspartate-aromatic aminotransferase isozymes I and II when supplied with D,L-phenylalanine (PHE) or a monochloro-D,L-phenylalanine

Substrate		Isozyme I		Isozyme II	
Keto acid*	Amino acid†	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)‡	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)‡
2-Oxoglutarate	Phe	17.4	100.0	38.0	100.0
	2Cl-Phe	0	0	12.9	34.0
	3Cl-Phe	9.2	52.4	32.3	85.0
	4Cl-Phe	21.7	123.9	125.4	330.0
Oxaloacetate	Phe	18.4	105.0	22.8	60.0
	2Cl-Phe	0	0	0	0
	3Cl-Phe	0	0	18.5	49.0
	4Cl-Phe	0	0	57.7	152.0
Glyoxylate	Phe	0	0	0	0
	2Cl-Phe	0	0	0	0
	3Cl-Phe	0	0	0	0
	4Cl-Phe	0	0	0	0
Pyruvate	Phe	0	0	0	0
	2Cl-Phe	0	0	0	0
	3Cl-Phe	0	0	0	0
	4Cl-Phe	0	0		

*All keto acids were tested at a final concentration of 10 mM.

†All amino acids were D,L-isomers tested at a final concentration of 40 mM.

‡The activity of isozyme I or II with each keto acid is expressed as a percentage of the activity observed with D,L-Phe as the amino acid and 2-oxoglutarate as the keto acid.

Table 3. L-Amino acid substrate specificity of bushbean aspartate-aromatic aminotransferase isozymes I and II

	Isozyme I		Isozyme II	
Substrate amino acid tested*	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†
L-Asp	6000.0	100.0	2812.0	100.0
L-Met	0	0	0	0
L-Ala	0	0	0	0
L-Val	0	0	0	0
L-Leu	0	0	0	0
L-Glu	0	0	0	0
L-Gln	0	0	0	0
L-Gly	0	0	0	0
L-Phe	40.8	0.68	90.0	3.2
L-Tyr	20.6	0.34	141.0	5.0
L-Trp	9.4	0.16	132.2	4.7

*All substrate amino acids were tested at a final concentration of 40 mM, except L-tyrosine and L-tryptophan which were assayed at 20 mM final concentration. Except with L-glutamate, the keto acid supplied was 2-OG in all cases, at a final concentration of 10 mM. When L-glutamate was tested as amino donor, OAA was supplied as the keto acid at a final concentration of 10 mM.

†The activity of each compound is expressed as a percentage of the activity of L-aspartate assayed at the same concentration.

tative members of each major family of amino acids based on their routes of biosynthesis [12]. The results, confirmed that both isozymes were multispecific aminotransferases capable of catalysing the transamination of aspartate as well as each of the three aromatic amino acids when 2-OG was supplied as the keto acid (Table 3). The rate of aromatic aminotransferase activity was low (0.1–5%) compared to that obtained with aspartate, and

glutamate was transaminated quite effectively when OAA was provided as the keto acid. None of the other L-amino acids tested were transaminated.

These results are very similar to those we reported earlier for two cytosolic isozymes of aspartate-aromatic aminotransferase purified from bushbean shoots [8] and to those obtained with a multispecific aspartate aminotransferase purified from bushbean roots [1]. Mammalian and bacterial aspartate aminotransferases have also been shown to exhibit similar multispecificity. Early studies with this enzyme isolated from pig heart tissue showed that while aspartate was certainly the primary substrate, aminotransferase activity ranging from 2 to 6% was also observed when any one of the three aromatic amino acids was supplied [13]. Later investigations with homogenous preparations of aspartate aminotransferase purified from rat liver [10] and rat heart [14] found the aromatic aminotransferase activity to be as high as 10–25% of that observed with aspartate. However, when the same enzyme was purified from rat brain tissue, its activity with the aromatic amino acids was much lower, being only 0.1–0.25% of that found with aspartate [15]. A multispecific aspartate aminotransferase, which preferred aspartate as its primary substrate, but also showed 10–13% activity when supplied with any of the aromatic amino acids was found in *E. coli* [4].

L-Aspartate was able to competitively inhibit the L-phenylalanine aminotransferase activity exhibited by a multispecific aspartate aminotransferase purified from bushbean roots [16]. This was confirmed in our earlier studies [8] where the L-phenylalanine aminotransferase activity of aspartate aminotransferase isozyme II from bushbean shoots was found to be inhibited up to 50% by physiological concentrations of L-aspartate. The results clearly suggest that both the aliphatic and aromatic amino acid substrates compete for the same active site on the multispecific isozyme II in bushbean shoots.

Thus, our earlier and present findings confirm that in bushbean, as in certain animals and bacteria, there are multispecific aspartate aminotransferases which show very similar substrate specificities. The structural similarities between aspartate and glutamate as substrates is apparent, but the fact that this enzyme also shows an affinity, albeit much reduced, for a group of amino acids as structurally different as the aromatic amino acids, yet shows little or no affinity for other aliphatic amino acids, seems very unusual at first glance. The keto acid substrate specificity of the enzyme generally favours 2-OG and OAA which, like glutamate and aspartate, are also 5- and 4-carbon dicarboxylates, respectively.

Since the properties of plant aminotransferases were last reviewed in this journal [5] some new details of the mechanism of action of these enzymes have come to light and are of particular relevance to the present study. The dynamic model for the mechanism of aminotransferase activity was proposed by Ivanov and Karpeisky [17] and reviewed by Braunstein [3] and by Metzler [18]. While the present model has arisen from studies of L-aspartate aminotransferase (EC 2.6.1.1) from animal sources, all the evidence currently available suggests that plant aminotransferases operate through a ping-pong bi-bi mechanism [19, 20] similar to that of animal aminotransferases [5, 21]. Recently, Arnone *et al.* [22, 23] and Kirsch *et al.* [24] have proposed stereochemical mechanisms for transamination which are progressive variations of the basic model of Ivanov and Karpeisky. The mechanisms are based on X-ray and microspectrophotometric studies of single crystals of L-aspartate aminotransferase purified from both cytosolic and mitochondrial fractions of pig heart.

While we are unaware of any detailed study on mechanism of action performed with a plant aminotransferase, it is known for animal aspartate aminotransferases that, for steric reasons, formation of the Michaelis complex must occur between the open conformation of the PLP-enzyme and the zwitterion form of the amino acid substrate [23]. The aspartate molecule is guided into the active site; the negative charge on the phenolate oxygen of the coenzyme attracts the protonated amine of aspartate, the positive charge on the guanidium group of Arg 386 in subunit 1 forms an ion pair with the α-carboxylate group of aspartate while the positive charge of Arg 292* in the neighbouring subunit forms a similar ionic interaction with the β-carboxylate group [23]. This results in changes in pK values of both the substrate and the coenzyme such that a proton can be transferred from the α-NH_3^+ group of the substrate to the imine nitrogen of the internal Schiff base [18]. The arrangement of the charges on the arginine residues and the shape of the pocket of the active site prevent non-productive binding and the binding of D-isomers of amino acids [24]. The active site cavity has strong steric constraints and only carboxylates with the *cisoid* eclipsed carboxylate group conformation can bind to aspartate aminotransferase [25].

It has been reported that the enhanced specificity for dicarboxylate substrates observed in animal aspartate aminotransferases is determined mainly by the interaction of these substrates with the Arg 386 and Arg 292* residues of the enzyme subunits [24]. The compensation of the charges on the arginine residues is thought to contribute to the conformational change of the PLP-enzyme from the open to the closed conformation, necessary for an efficient aldimine to ketimine conversion [24]. In this connection, a comparative study of the amino acid sequences of several aspartate aminotransferase isozymes purified from animal cytosolic and mitochondrial fractions, and from *E. coli*, showed that all isozymes had positively-charged arginine residues at 292* and at 386 [26].

Thus, the ability of aspartate aminotransferases from various sources to make limited use of the aromatic amino acids as substrates may well lie in the affinity of the electron-rich aromatic ring for the positively-charged area (Arg 292*) of the active site normally occupied by the distal carboxylate group of aspartate or glutamate. The degree to which this important 'ion-pairing' can occur will affect the degree to which the enzyme can assume a closed conformation and subsequently, the efficiency of the aldimine to ketimine transition. The importance of ring structure on aminotransferase activity will become more apparent in the next section discussing the results of substrate specificity studies with the bushbean isozymes I and II using a range of substituted aromatic amino acids.

Substrate specificity studies with substituted aromatic amino acids

Isomer and sidechain substitution effects. The aminotransferase activities of isozymes I and II when different substituted aromatic amino acids were provided as the substrate are shown in Tables 4–6. Although most of the substituted acids tested were supplied in the D,L-form, whenever the two isomers were examined separately,

Table 4. Substrate specificity of bushbean aspartate-aromatic aminotransferase isozymes I and II for a series of substituted phenylalanine (Phe) analogues

Substrate amino acid tested*	Isozyme I		Isozyme II	
	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†
Parent compound				
L-Phe	41.0	254.0	93.6	227.0
D-Phe	0	0	0.5	1.4
D,L-Phe	16.0	100.0	33.9	100.0
Sidechain-substituted				
N-Acetyl-L-Phe	0	0	0	0
N-Chloroacetyl-L-Phe	0	0	0	0
N-Chloroacetyl-D,L-Phe	0	0	0	0
N-Carbobenzoxy-L-Phe	0	0	0	0
α-Methyl-L-Phe	0	0	0	0
Ring-substituted				
2-Fluoro-D,L-Phe	0	0	3.3	9.5
3-Fluoro-D,L-Phe	0	0	7.2	21.7
4-Fluoro-D,L-Phe	0	0	21.6	64.0
2-Chloro-D,L-Phe	0	0	6.3	19.0
3-Chloro-D,L-Phe	5.6	35.0	16.5	49.0
4-Chloro-D,L-Phe	11.5	72.0	88.8	263.0
4-Bromo-D,L-Phe	10.2	64.0	54.0	316.0
4-Iodo-D,L-Phe	6.7	42.0	45.3	263.0
4-Amino-D,L-Phe	25.9	162.0	158.1	468.0
4-Nitro-D,L-Phe	0	0	0	0
2-Naphthylalanine	0	0	0	0
Sidechain and ring-substituted				
Acetyl-2-fluoro-D,L-Phe	0	0	0	0
Acetyl-3-fluoro-D,L-Phe	0	0	0	0
α-Methyl-3-methoxy-D,L-Phe	0	0	0	0

*All substrate amino acids were tested at a final concentration of 40 mM, except the 4-bromo- and 4-iodo compounds which were tested at a final concentration of 20 mM, due to limited solubility. The keto acid supplied in each case was 2-OG at a final concentration of 10 mM.

†The activity of each compound is expressed as a percentage of the activity of the corresponding isomer of the unsubstituted parent compound assayed at an equivalent concentration.

activity with the D-isomer was either absent or very low both with the parent amino acid and with substituted derivatives. This can be seen, for example, by comparing the activities of the D- and L-isomers of phenylalanine (Table 4), tyrosine and 3,4-dihydroxyphenylalanine (DOPA) (Table 5) and of tryptophan and 5-hydroxytryptophan (Table 6). With the parent amino acids, the D,L-isomer was generally *ca* 40–50% less active than the L-isomer when assayed at an equivalent concentration, which confirmed that the purification of isozymes I and II had resulted in the isolation of L-specific forms of each enzyme. Compounds containing substituents in the sidechain which included *N*-acetyl-, *N*-chloroacetyl-, *N*-carbobenzoxy-, *O*-carbobenzoxy-, or α-methyl- groups, were all found to be inactive (Tables 4–6). Substitution at the α-carboxyl group would prevent the important ionic pairing with a strongly positive amino acid residue close to the coenzyme (such as Arg 386) as discussed above. Substitution at the nitrogen of the amino group would prevent the latter from reacting at the active site on the aminotransferase, since the lack of a protonated amino group would prevent formation of the transaldimination intermediate. Thus, the *N*-substituted amino acids would not be expected to have aminotransferase activity. The α-H atom also plays an essential role in the aminotransferase mechanism since it is removed from the α-C of the aldimine form of the Schiff's base prior to the formation of the tautomeric ketimine form of the complex. Therefore, as might be expected, α-methyl substituted derivatives were also found to be inactive as substrates for the bushbean isozymes I and II (Tables 4–6). Sidechain substitution accompanied by ring substitution completely abolished any activity observed as a result of ring substitution alone. For example, *N*-acetylation of 2- and 3-fluoro-substituted D,L-phenylalanines resulted in complete loss of the activity observed with these ring-substituted phenylalanines (Table 4). α-Methylation of the sidechain of 3,4-dihydroxy-L-phenylalanine also abolished the activity shown by this compound (Table 5).

Ring-substituted phenylalanine analogues. Substrate specificity studies with a series of ring-substituted phenylalanines showed that many analogues were transaminated

Table 5. Substrate specificity of bushbean aspartate-aromatic aminotransferase isozymes I and II for a series of substituted tyrosine (Tyr) analogues

Substrate amino acid tested*	Isozyme I		Isozyme II	
	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†
Parent compound				
L-Tyr	20.0	190.0	156.0	222.0
D-Tyr	0	0	5.0	1.4
D,L-Tyr	10.5	100.0	70.0	100.0
Sidechain-substituted				
O-Carbobenzoxy-L-Tyr	0	0	0	0
N-Acetyl-L-Tyr	0	0	0	0
N-Chloroacetyl-L-Tyr	0	0	0	0
N-Carbobenzoxy-L-Tyr	0	0	0	0
Ring-substituted				
D,L-*o*-Tyr	0	0	0	0
D,L-*m*-Tyr	0	0	16.0	23.0
3,4-Dihydroxy-L-Phe	7.0	35.0	73.0	47.0
3,4-Dihydroxy-D-Phe	0	0	0	0
3-Iodo-L-Tyr	7.6	38.0	88.0	56.0
3-Amino-L-Tyr	3.0	15.0	45.0	29.1
3-Nitro-L-Tyr	0	0	10.55	6.7
3,5-Diiodo-L-Tyr	0	0	0	0
3,5-Dibromo-L-Tyr	0	0	5.0	3.2
3,5-Dinitro-L-Tyr	0	0	0	0
Sidechain and ring-substituted				
α-Methyl-D,L-*m*-Tyr	0	0	0	0
α-Methyl-D,L-Tyr	0	0	0	0
α-Methyl-3,4-dihydroxy-D,L-Phe	0	0	0	0

*All substrate amino acids were tested at a final concentration of 20 mM. The keto acid supplied in each case was 2-OG at a final concentration of 10 mM.

†The activity of each compound is expressed as a percentage of the activity of the corresponding isomer of the unsubstituted parent compound assayed at an equivalent concentration.

by both isozymes I and II (Table 4). Isozyme II was able to utilize all halogenated phenylalanines as substrates and especially the amino-substituted derivative. With the fluoro- and chloro-substituted analogues, isozyme II showed an increasing ability to transaminate the amino acid as the ring-substituent shifted from the 2- to 3- to 4-position. 4-Chloro-D,L-phenylalanine was transaminated at a rate which was more then 2.5-fold higher than that obtained with the parent D,L-phenylalanine. The 4-bromo- and 4-iodo-D,L-phenylalanines were also transaminated at rates 2.5–3 times higher than that observed with D,L-phenylalanine. Thus, halogen substitution in the 4-position led to a very significant increase in aminotransferase activity. 4-Amino-D,L-phenylalanine showed the highest activity of all the analogues examined, with a rate of transamination approaching five times that shown by the parent D,L-amino acid. In contrast, 4-nitro-D,L-phenylalanine was completely inactive as a substrate.

Isozyme I was comparatively much less reactive with the ring-substituted phenylalanine compounds. None of the fluoro-substituted or the 2-chloro-substituted phenylalanines were transaminated (Table 4). Halogenation in the 4-position resulted in good rates of transamination (40–75%) but these were always less than that observed with D,L-phenylalanine. Only 4-amino-D,L-phenylalanine was transaminated at a higher rate (1.6-fold) by isozyme I than that observed with the unsubstituted amino acid. Again, 4-nitro-D,L-phenylalanine was inactive.

With regard to the influence of ring systems larger than phenyl- or indolyl- on the activity of bushbean isozymes I and II, 2-naphthylalanine was found to be inactive with both isozymes suggesting that there are steric constraints limiting the allowable size of the aromatic ring system at the active site.

In studies with animal aminotransferases, 4-amino-phenylalanine was about twice as active as L-phenylalanine as a substrate for the tyrosine aminotransferase from rat liver [9], but in contrast to the present results with a comparable plant aminotransferase (Table 4), these earlier workers found that 4-fluorophenylalanine was twice as active as L-phenylalanine as a substrate for the rat liver enzyme. Miller and Litwack [10] reported that 4-chlorophenylalanine was quite poorly transaminated by a purified rat liver tyrosine aminotransferase, supporting a rate of activity only about 10% of that shown by L-phenylalanine; the 4-chloro- analogue was not metabolised at all when supplied to a rat liver cytosolic preparation [11]. 2-Naphthylalanine does not appear to have been tested with any animal aminotransferase preparation.

Table 6. Substrate specificity of bushbean aspartate-aromatic aminotransferase isozymes I and II for a series of substituted tryptophan (Trp) analogues

Substrate amino acid tested*	Isozyme I		Isozyme II	
	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†	Sp. activity (units/mg protein $\times 10^{-3}$)	Relative activity (%)†
Parent compound				
L-Trp	9.0	188.0	135.0	209.0
D-Trp	0	0	0	0
D,L-Trp	4.8	100.0	64.6	100.0
Sidechain-substituted				
N-Acetyl-L-Trp	0	0	0	0
N-Acetyl-D,L-Trp	0	0	0	0
N-Chloroacetyl-L-Trp	0	0	0	0
N-Carbobenzoxy-L-Trp	0	0	0	0
α-Methyl-D,L-Trp	0	0	0	0
Ring-substituted				
5-Hydroxy-L-Trp	3.6	40.0	48.5	36.0
5-Hydroxy-D-Trp	0	0	0	0
5-Hydroxy-D,L-Trp	1.9	39.6	23.0	35.6
5-Methyl-D,L-Trp	0.5	10.4	12.4	19.0
5-Benzyloxy-D,L-Trp	0	0	0	0

*All substrate amino acids were tested at a final concentration of 20 mM. The keto acid was 2-OG in all cases, at a final concentration of 10 mM.
†The activity of each compound is expressed as a percentage of the activity of the corresponding isomer of the unsubstituted parent compound assayed at an equivalent concentration.

Thus, the present results with ring-substituted phenylalanines suggest that the bushbean aspartate aminotransferase isozymes I and II may have slightly different binding site properties with respect to each other and also with respect to the binding sites in multispecific animal aspartate aminotransferases. These differences will be considered in the final section of the discussion.

Ring-substituted tyrosine analogues. With a series of tyrosine analogues, none of the ring-substituted compounds were found to serve as better substrates than the parent amino acid when tested with the bushbean isozymes I and II (Table 5). Both L-and D,L-tyrosine were readily transaminated by both isozymes, with isozyme II showing a level of activity with the L-isomer which was 50% higher than that observed earlier with L-phenylalanine (Table 4). Isozyme II also showed a broader range of specificity than isozyme I with the tyrosine analogues, which was similar to the trend observed with the phenylalanine analogues. The highest activities shown by isozymes I and II with the ring-substituted tyrosines were found with the L-isomers of 3-iodo- and 3,4-dihydroxy-tyrosines (Table 5). As expected, the D-isomer of 3,4-dihydroxy-phenylalanine was inactive. While 3-iodotyrosine was moderately active with both isozymes, the 3,5-diiodo-analogue was inactive. 3,5-Dinitrotyrosine was also inactive with both isozymes, although small levels of activity were observed with isozyme II in presence of the 3-nitro- and 3,5-dibromo-analogues.

In comparable studies with animal aromatic aminotransferases, although *m*-tyrosine was transaminated at about 25% of the rate shown by the natural *p*-tyrosine, the *ortho* analogue gave very low rates of activity when the three amino acids were tested as substrates for a mitochondrial enzyme system from rat liver [11]. A purified mitochondrial tyrosine aminotransferase from rat liver was able to transaminate 3,4-dihydroxy-L-phenylalanine (L-DOPA) at 85% of the rate obtained with L-tyrosine [10], while the corresponding purified cytosolic enzyme was only able to transaminate the dihydroxy-compound at a rate 10% of that shown by L-tyrosine [27]. 3-Iodo-L-tyrosine has also been shown to be a good substrate for mammalian tyrosine aminotransferases where activities of 100 and 84% of those found with L-tyrosine have been reported using a rat liver cytosolic enzyme [9] and a rat liver mitochondrial enzyme [10]. In contrast, the di-substituted analogue, 3,5-diiodotyrosine, was found to be inactive as a substrate for the rat liver cytosolic tyrosine aminotransferase [9], which agrees with the result obtained in the present study when this compound was tested as a substrate for the bushbean isozymes I and II (Table 5). However, a halogen-activated tyrosine aminotransferase in mitochondria from rat liver which catalysed the transamination of 3,5-diiodo-tyrosine, 3-iodotyrosine and tyrosine in the ratio 100:77:4 has been found [28]. The enzyme was also shown to utilise the thyroid hormone thyroxine and to a lesser extent, phenylalanine and tryptophan. In general, however, the ability of the bushbean isozymes I and II to utilize tyrosine analogues is similar to that found with corresponding mammalian aspartate aminotransferases; L-tyrosine is still the best substrate of the hydroxy-substituted aromatic compounds examined thus far.

Ring-substituted tryptophan analogues. The relative activities of the substituted tryptophan derivatives with the bushbean isozymes I and II are shown in Table 6. Only certain ring-substituted analogues were able to serve as substrates for both isozymes and all sidechain-substituted analogues were completely inactive as reported earlier. 5-Hydroxy-L-tryptophan was transaminated by both isozymes at 35–40% of the rate shown by L-tryptophan, but the corresponding 5-methyl-derivative was only able to support 10–20% of the activity shown by the parent amino acid. As in the tyrosine series, the highest activity was again obtained with the natural amino acid, L-tryptophan.

In previous studies with this class of compounds, Jacoby and LaDu [9] were unable to demonstrate any aminotransferase activity towards 5-hydroxy-L-tryptophan in rat liver cytosolic systems, although earlier workers had obtained some evidence for the transamination of this compound in rat liver tissue [29, 30]. An aromatic aminotransferase from the micro-organism *Achromobacter eurydice*, which was active with all three aromatic amino acids and could also use 5-hydroxy-L-tryptophan as a substrate has been described [31].5-Methyl-L-tryptophan showed 39% of the activity of L-tryptophan when tested in a rat liver cytosolic system [9], which is greater than the activity obtained with this substituted amino acid when supplied to the bushbean isozymes (Table 6).

Trends in the structure–activity relationships of substituted amino acids with the bushbean isozymes I and II

From the activities observed with the bushbean aminotransferase isozymes I and II when substituted aromatic amino acids were provided as substrates (Tables 4–6), the following trends are apparent in the structure–activity relationships of these compounds:

(1) All side chain-substituted compounds are totally inactive. (2) Ring substituted phenylalanines are by far the most reactive substrates, whereas substitution in the ring of tyrosine or tryptophan results in lower activity than that observed with the parent amino acid. (3) All monohalogen ring-substituted phenylalanines are reactive, with the *para*-halogenated compounds being very active substrates. (4) *para*-Aminophenylalanine shows the highest activity of all the substituted phenylalanines tested, whereas *para*-nitrophenylalanine is completely inactive. (5) Isozyme II exhibits greater breadth of multispecificity than isozyme I and shows much greater activity with the *para*-halogen-, *para*-hydroxy- and *para*-aminophenylalanines. (6) Trends 3–5 indicate that both electronic (ring resonance and/or induction) and steric effects play a role in establishing the efficiency with which the aromatic moiety of the amino acid binds at the active site on isozyme II.

Proposed model for activation of aromatic ring binding to the 'distal carboxylate' site of isozyme II by certain ring substituents

If one assumes that the active sites of the bushbean aminotransferase isozymes I and II are similar to that of multispecific animal aspartate aminotransferases (as discussed above), then the binding of the substrate amino acid must compensate two positive charges on the PLP-enzyme; one (e.g. Arg 386).forms an ion pair with the α-carboxylate group, while the other (e.g. Arg 292* of subunit 2) binds the distal carboxylate group of aspartate or glutamate or the aromatic ring of phenylalanine, tyrosine or tryptophan.

The present study shows that *para*-amino, *para*-hydroxy or *para*-halogen substitution in the phenylalanine ring leads to enhanced substrate activity over that shown by the unsubstituted parent amino acid, while *para*-nitro substitution exerts the opposite effect. The contribution of the nature and position of the ring substituent to electronic effects during aromatic substrate binding can be readily envisioned if one considers that the effect of compensating the positive charge at Arg 292* by ion pairing may be analogous to an electrophilic aromatic substitution reaction; that is, the electrophilic arginine 292* residue 'adds to' the electron rich aromatic ring of the substrate amino acid. As outlined in ref. [32] substituents found on aromatic rings can be classified as strong and weak activators or deactivators of electrophilic aromatic substitution (Table 7). If a group takes on a negative charge by induction, the group is said to possess a −I effect; if a group takes on a positive charge by induction, it is said to possess a +I effect. In a similar manner, if the group takes on a negative charge by resonance, it possesses a −R effect, while if it takes on a positive charge by resonance, it is said to possess a +R effect. A +I or +R effect tends to make the substituent positive and the ring negative, and a −I or −R effect tends to put a negative charge on the substituent and a positive charge in the ring [32].

These same substituent groups can also be classified according to their positional directing effects on electrophilic aromatic substitution [32]. A *para*-amino group or *para*-hydroxyl group substituted in phenylalanine is a powerful activator for *ortho*, *para*-substitution (Table 8). The *para* position of the phenyl ring in such monosubstituted analogues, however, is already occupied by the alkyl amino acid sidechain, which is a comparatively weak *ortho*, *para* directing activator of electrophilic aromatic substitution. Therefore, the strong activating $-NH_2$ and -OH groups dominate and become powerful *ortho* directors. In contrast, the *para*-nitro group substituted in phenylalanine is a powerful deactivator and *meta* director. The halogens are unusual in their effects on electrophilic aromatic substitution; they are weakly deactivating yet *ortho*, *para*-directing. While a *para*-halogen atom must compete with the weakly activating *ortho*, *para*-directing effect of the alanine sidechain, steric considerations

Table 7. Classification of ring substituent groups according to their resonance (R) and induction (I) effects on electrophilic aromatic substitution (modified from ref. [32])

Activating groups	Deactivating groups
Strong	
$-NH_2$, $-NHR$, $-NR_2$,	$-NO_2$ (−R, −I)
−OH, $-O^-$ (+R)	$-NR_3^+$ (−I)
Intermediate	
−OR, −NHCOR (+R, −I)	−C≡N, −CHO, −COR
	−COOH (−R, −I)
Weak	
−Ph (+R)	−F, −Cl, −Br, −I (+R, −I)
−Alkyl (+R, +I)	

Table 8. Classification of ring substituent groups according to their positional directing effects on electrophilic aromatic substitution (modified from ref. [32])

Ortho, Para Directors	*Meta* Directors
Strong	
$-NH_2$, $-NHR$, $-NR_2$, $-OH$, $-O^-$	$-NO_2$, $-NH_3^+$, $-NR_3^+$
Intermediate	
$-OR$, $-NHCOCH_3$	$-C{=}N$, $-SO_3H$
Weak	
$-R$, $-Ph$, $-F$, $-Cl$, $-Br$, $-I$	$-CHO$, $-COR$, $-COOR$, $-COOH$

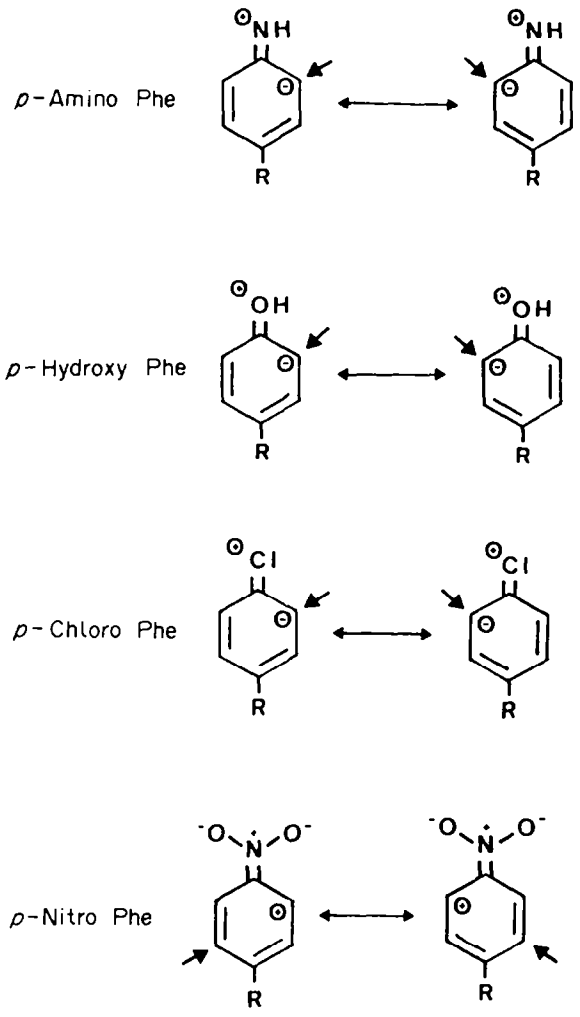
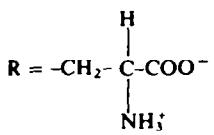


Fig. 1. Favoured canonical resonance forms of certain *para*-substituted phenylalanines for ion pairing with an electrophile. Probable sites of attack by an electrophile (e.g. Arg 292*) are indicated by the large arrows.



suggest an easier approach for the Arg 292* electrophile to a position *ortho*- to the halogen, than to one *ortho*- to the large alkyl sidechain.

The common property linking the halogenated, aminated and hydroxylated phenylalanines, which show high activity with isozyme II, is that all of the substituents are '+R', that is, they take on a positive charge by resonance (Table 7) and are also all *ortho, para* directing (Table 8). This suggests that the canonical resonance forms shown in Fig. 1 will favour *ortho* attack by the electrophile (e.g. Arg 292*). Thus, ion pairing between the positive enzyme residue and the aromatic ring will be strongest when the ring position, *ortho* to the substituent, is able to 'line up' with the positive charge at Arg 292* on the enzyme.

A spatial model for the active site of chicken heart aspartate aminotransferase [33] suggested that both aspartate and glutamate adjust conformationally to fit a site 6.2 Å wide, with the α- and distal carboxylate groups in a *cisoid* conformation. Figures 2(a) and (b) show the conformation in which aspartate is thought to bind at the active site. Assuming that the aromatic rings of phenylalanine and tyrosine must somehow adjust to this same binding site for enzyme activity, we have constructed models of L-phenylalanine and L-tryosine with various ring substituents. It appears from the models of phenylalanine and tyrosine that only when the *ortho, para* directing '+R' groups are in the 4-position on the ring, can both of these conditions be fulfilled, giving a molecule whose α- and resonance-induced distal negative charges

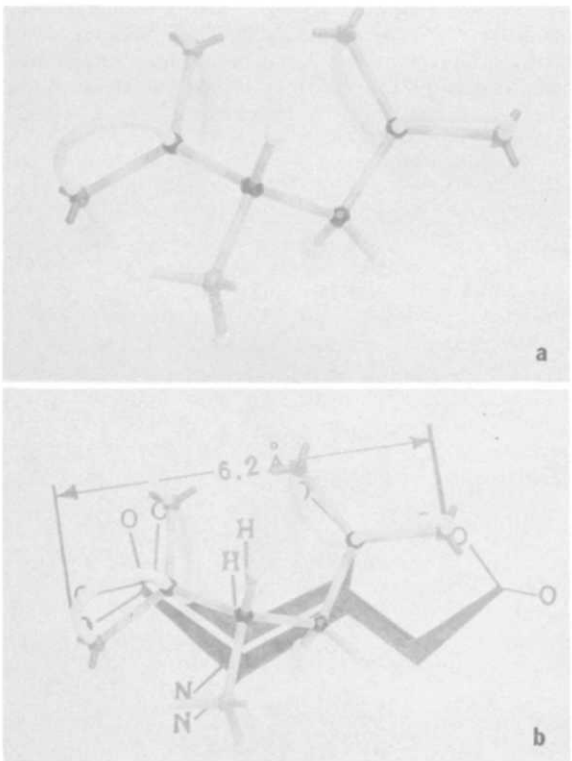


Fig. 2. (a). Suggested spatial orientation of L-aspartate required for entry into the substrate binding site of the PLP-enzyme complex. (b) This orientation allows L-aspartate to fit the binding site dimensions as proposed in ref. [33], in which the α- and distal carboxylate groups are separated by 6.2 Å.

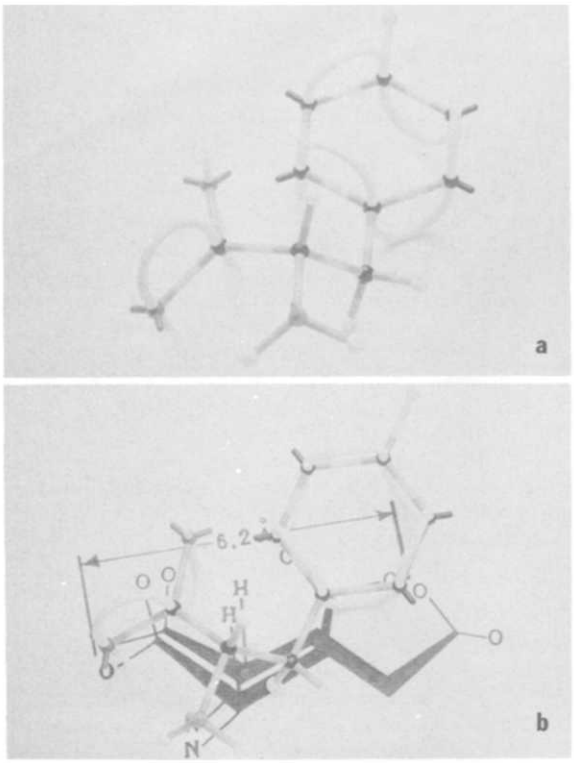


Fig. 3. (a). Suggested spatial orientation of L-4-chlorophenylalanine required for entry into the substrate binding site of the PLP-enzyme complex. (b) This orientation would allow L-4-chlorophenylalanine to fit the binding site dimensions proposed in ref. [33], in which the α- and resonance-induced ring charges are separated by 6.2 Å.

are 6.2 Å apart and *cisoid* in terms of approach to the binding site. Figures 3(a) and (b) demonstrate how 4-chlorophenylalanine, with its resonance-induced *cisoid* negative charges, is able to accommodate these binding site restrictions.

When steric hindrance to free rotation and to the approach of the substrate to the active site were considered, we utilized an enlargement of a spatial model [24] for the Michaelis complex of the PLP-enzyme with aspartate (closed conformation) to try to 'fit' the various models to the active site. For example, Fig. 4 shows the interaction between the *cisoid* eclipsed conformation of aspartate and Arg 292*. Activating substituents in the *ortho*- or *meta*- position on the phenylalanine ring resulted in steric hindrance either to the approach of the substrate to the PLP-site or hindrance to a *cisoid* approach of the α- and distal negative charges to Arg 386 and Arg 292*, and so were even less reactive than the unsubstituted parent compound. With *para*-substituents, however, this did not appear to be the case. In fact, as compared to an unsubstituted ring system, *para*-substitution resulted in an enhanced ability to form an ion pair with Arg 292* due to its *ortho*-directing effects, as shown in Fig. 5 for 4-chlorophenylalanine. Models of 4-hydroxy- and 4-amino-phenylalanine were able to interact with the binding site in a similar manner. A model of 4-nitrophenylalanine, on the other hand, was unable to fit into the binding site because of steric hindrance and repulsion due to its resonance-induced *ortho* positive charges.

Di-substitution of the aromatic ring always resulted in a decrease in reactivity of the amino acid substrate as compared to the parent phenylalanine or tyrosine (Tables 4 and 5). This presumably is due to an increase in steric hindrance at the binding site, complicated by a less favourable distribution of 'directing effects' for ion pair formation.

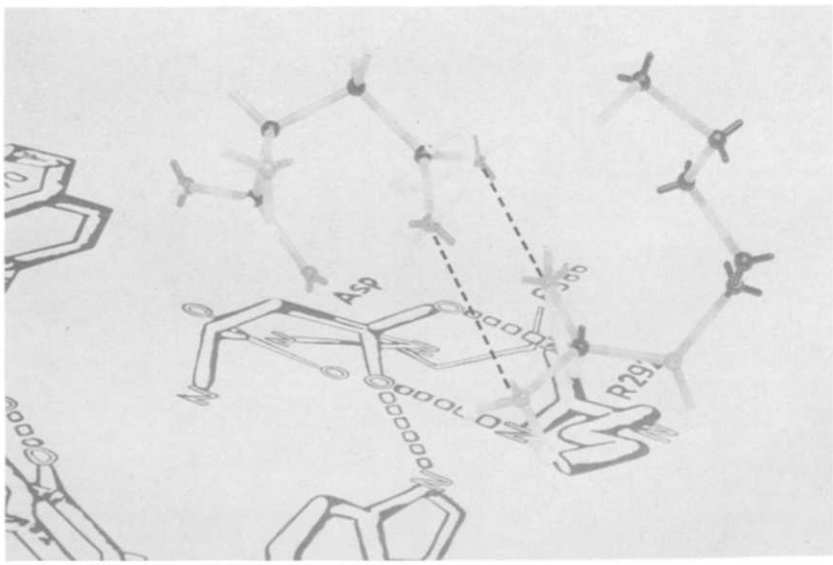


Fig. 4. Molecular model of L-aspartate shown interacting with the Arg 292* residue at the substrate binding site of aspartate aminotransferase. The substrate and the arginine residue have been superimposed on an enlargement of the spatial model for the Michaelis complex of the PLP-enzyme with aspartate (closed conformation) [24]. Ion pairing is indicated by the dashed lines.

In the present model, the binding of unsubstituted tryptophan at the active site would involve the indolyl ring at the C-7 position. Substitution of the molecule at the 5-position with any substituent would pose a considerable degree of steric hindrance. If the substituent is another strong *ortho, para* activator, such as a hydroxyl group, it would have a directing effect toward electrophilic attack at the C-6, C-7 carbons, thereby leading to a conflict between strong positional directing effects. In concert with steric hindrance, the predictable result would be a decrease in activity compared to that of the unsubstituted parent amino acid. Aromatic rings larger than the indolyl group are clearly sterically unfavourable in a similar way and the inactivity of 2-naphthylalanine with both bushbean isozymes supports this conclusion.

As might be expected, there are reservations to the present model. First, it assumes that the active site of multispecific aspartate aminotransferases in plants has two positively-charged, ion-pair-forming residues which are analogous to the Arg 386 and Arg 292* known to form part of the active site of mammalian and bacterial aminotransaminases [26]. Whether this is, in fact, the case must await detailed studies with a crystalline plant aspartate aminotransferase comparable to those performed over the last decade with crystalline animal aspartate aminotransaminases (see [34]). The model also assumes that a *para*-substituent does not hinder the approach of the aromatic ring to the 'distal' positive binding site on isozyme II. Kirch et al. [24] have proposed that during the aldimine to ketimine transition of the aminotransferase reaction, the hydrogen bond between the distal-carboxylate and the Arg 292* residue can remain intact due to the high degree of flexibility of the arginine sidechain. Indeed, as shown in Fig. 5, such flexibility may in fact accommodate substrates by forming a pocket into which sterically-favourable substituents can fit. This may be a limiting factor for some enzymes but not for others. For example, bushbean isozyme I does not exhibit the same high activities with the *para*-halogenated phenylalanines as that shown by bushbean isozyme II. This may indicate that in isozyme I there are steric constraints imposed by the topography of the active site which could prevent an enhanced binding of 4-chlorophenylalanine over that experienced by unsubstituted L-phenylalanine.

In summary, interesting trends in electronic and steric influences on the reactivity of ring-substituted aromatic amino acids with the active site of aspartate-aromatic aminotransferases are evident from the present examination of the substrate multispecificity of these enzymes purified from plant, animal and bacterial sources. It is hoped that future investigations in this area will further improve our understanding of the mechanism by which the active site of this multispecific enzyme can accommodate such structurally different substrates as aspartate, glutamate and the aromatic amino acids.

EXPERIMENTAL

Plant material. The shoots of 10-day-old light-grown seedlings of bushbean (*Phaseolus vulgaris* L. var Pencil Pod Black Wax) were used as the plant material for preparation of the purified enzyme fractions. Seeds were soaked in distilled water at room temp. overnight, sown in moist vermiculite, germinated in a growth cabinet at 25/22° day/night temperatures and exposed to a 16 hr daily photoperiod of 4600–4800μW/cm^2. Time of planting was taken as day 0. Seedlings were harvested at the 10-day stage and epicotyl tissue collected for enzyme extraction.

Chemicals and chromatography supplies. All chemicals used in this investigation were of analytical grade. Most of the substituted aromatic amino acid substrates were purchased from Nutritional Biochemicals Corporation with the following exceptions, which were all obtained from the Sigma Chemical Corporation: D,L-3-fluorophenylalanine, *N*-carbobenzoxy-L-phenylalanine, D,L-4-iodophenylalanine, *N*-acetyl-L-

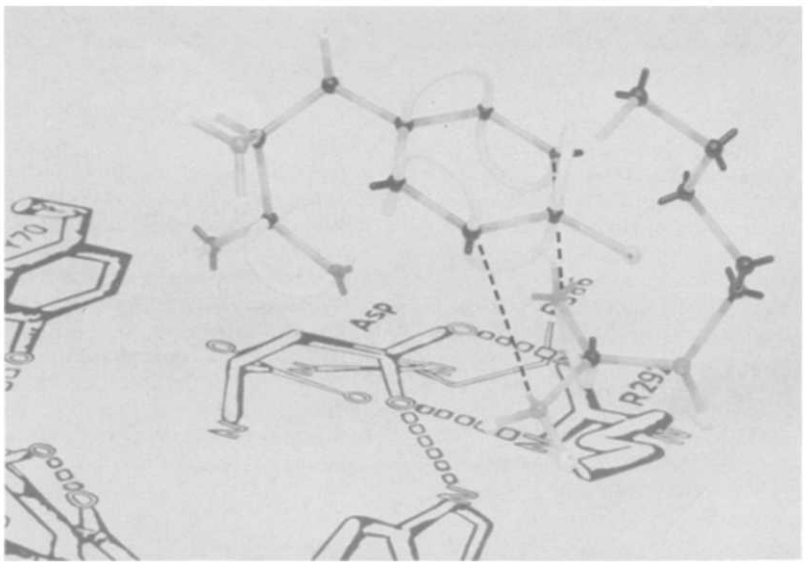


Fig. 5. Molecular model showing proposed interaction between L-4-chlorophenylalanine and the Arg 292* residue at the substrate binding site of aspartate aminotransferase. The 4-chlorophenylalanine and the arginine residue have been superimposed on an enlargement of the spatial model for the Michaelis complex of the PLP-enzyme with aspartate (closed conformation) [24]. Ion pairing is indicated by the dashed lines.

phenylalanine, *N*-chloroacetyl-L-phenylalanine, L-tryptophan, D-tryptophan, α-methyl-D,L-tryptophan, *N*-carbobenzoxy-L-tryptophan, *N*-acetyl-L-tyrosine, L-3-nitrotyrosine, L-3-iodotyrosine, L-3-aminotyrosine, α-methyl-D,L-3-tyrosine, *N*-chloroacetyl-L-tyrosine, *N*-carbobenzoxy-L-tyrosine, L- and D-3,4-dihydroxyphenylalanine and α-methyl-D,L-3,4-dihydroxyphenylalanine. The purity of each aromatic amino acid was checked by chromatography on large sheets (46 × 57 cm) of Whatman No. 1 paper. Chromatograms were developed in the descending manner for 12 hr in *n*-BuOH–HOAc–H_2O (90:10:29), and the amino acids visualized with a modified $Cd(OAc)_2$–ninhydrin colorimetric reagent using the method of ref. [35]. In most cases, only a single ninhydrin-positive spot was observed on the chromatogram. Those compounds which contained trace impurities were purified by re-crystallization from 10% MeOH.

Sephadex G-25 (fine), Sephacryl S-300 and DEAE-Sephacel were purchased from Pharmacia, while Biogel HTP (hydroxylapatite) was purchased from Bio-Rad Laboratories.

Purification of cytosolic aspartate-aromatic aminotransferase isozymes I and II. 65 g of shoot material were rinsed in ice-cold distilled H_2O, chopped into fine pieces and then ground with a pestle and mortar for 3–5 min in 200 ml of an ice-cold grinding medium modified from ref. [36], containing 400 mM sucrose, 50 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 10 mM KCl, 10 mM EDTA, 2 mM DTT, 8 mM 2-OG, 40 mM 2-mercaptoethanol and 5% glycerol. After filtering through 8 layers of cheesecloth, the homogenate was centrifuged at 2000 *g* for 10 min to sediment chloroplasts and cell debris. The supernatant was centrifuged at 15000 *g* for 10 min, the pellet fraction containing mitochondria was discarded and the supernatant was then centrifuged a final time at 100000 *g* for 120 min to pellet the microsomal fraction. From the resulting supernatant enriched in cytosolic components, two isozymes of aspartate-aromatic aminotransferase were resolved and purified by a procedure involving a 40–70% $(NH_4)_2SO_4$ protein fractionation step followed by gel filtration of this fraction on Sephacryl S-300 and ion exchange chromatography on DEAE-Sephacel at pH 6.8. The latter step resolved the L-phenylalanine aminotransferase activity into two isozymic fractions and each enzyme was further purified by hydrophobic chromatography on hydroxylapatite. Isozyme II was then subjected to a final ion exchange purification on DEAE-Sephacel at pH 8.10. Details of this purification protocol and of the gel electrophoretic procedure for examining the purity of the final isozyme preparations, have been reported previously [7].

Protein determinations. During enzyme purification, the protein in column eluate fractions was monitored by A_{280} [37]. Total protein present at each purification stage and in the final isozyme I and II preparations was measured by the method of ref. [38].

Assay of aminotransferase activity. L-Phenylalanine aminotransferase activity was monitored during each purification step by measuring the production of phenylpyruvate at 318 nm, according to the method of ref. [39], as previously described [7].

Aminotransferase activity with the purified isozyme I and II preparations was measured by determining the amount of product amino acid produced in each reaction system after a 1–3 hr incubation period. The method of *o*-phthalaldehyde (OPA) derivatization followed by HPLC was used to isolate and quantify the OPA-amino acid derivative as previously described [7]. A typical 0.75 ml reaction system contained the following components dissolved in 50 mM Tris–HCl buffer, pH 8.5: 20–40 mM amino acid substrate, 10 mM keto acid, 0.1 mM PLP and 100 μl of isozyme I (*ca* 6 μg protein) or isozyme II (*ca* 15 μg protein). Due to solubility difficulties, each of the substituted aromatic amino acid substrates was prepared in 50 mM Tris–HCl, pH 8.5 containing 12.5% DMSO. Final concentration of DMSO in the assay reaction mixtures was always 10% and for comparative purposes, equivalent complete reaction mixtures containing the unsubstituted L- or D,L-aromatic amino acid plus DMSO were run simultaneously. The presence of DMSO was found to have no deleterious effect on L- or D,L-phenylalanine aminotransferase activity. Control reactions contained all of the above components except the amino acid substrate which was replaced by an equal vol. of Tris buffer. All reaction and control systems were incubated for 1 hr (L-amino acid substrate specificity studies) or for 3 hr (substituted aromatic amino acid substrate specificity studies) at 35° and each reaction was stopped by the addition of 100 μl of 30% sulphonyl salicylic acid soln. The precipitated protein was pelleted by centrifugation and the primary reaction product, glutamate, present in the supernatant was separated and measured by OPA-HPLC [7].

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