Exploring the Active Site of Amine:Pyruvate Aminotransferase on the Basis of the Substrate Structure−Reactivity Relationship: How the Enzyme Controls Substrate Specificity and Stereoselectivity

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An active site model of the amine:pyruvate aminotransferase (APA) from *Vibrio fluvialis* JS17 was constructed on the basis of the relationship between substrate structure and reactivity. Due to the broad substrate specificity of the APA, various amino donors (chiral and achiral amine, amino acid, and amino acid derivative) and amino acceptors (keto acid, keto ester, aldehyde, and ketone) were used to explore the active site structure. The result suggested a two-binding site model consisting of two pockets, one large (L) and the other small (S). The difference in the size of each binding pocket and strong repulsion for a carboxylate in the S pocket were key determinants to control its substrate specificity and stereoselectivity. The L pocket showed dual recognition mode for both hydrophobic and carboxyl groups as observed in the side-chain pockets of aspartate aminotransferase and aromatic aminotransferase. Comparison of the model with those of other aminotransferases revealed that the L and S pockets corresponded to carboxylate trap and side-chain pocket, respectively. The active site model successfully explains the observed substrate specificity as well as the stereoselectivity of the APA.

Introduction

The use of enzymes has emerged as a powerful tool in organic synthesis during the past decade and has spurred the development of biocatalytic processes for production of enantiomerically pure compounds.[1] The number of enzymes employed in organic synthesis is rapidly growing, which enriches the organic chemist's toolbox. However, it is often difficult to design a general enzymatic strategy for a given reaction due to delicate substrate specificity of the enzyme. Therefore, it is essential to precisely understand a substrate recognition mechanism and to construct an active site model for rapid determination of whether a compound of interest can be used as a substrate by the enzyme.

Aminotransferase is well characterized pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes reversible transfer of an amino group between amino acid and keto acid.[2] The overall reaction can be divided into two half reactions: (1) an amino acid (amino donor) donates its amino group to PLP form of the enzyme (E-PLP) to produce enzyme−pyridoxamine 5′-phosphate (E-PMP) and the corresponding keto acid, or (2) a different keto acid (amino acceptor) accepts the amino group from the E-PMP to produce the corresponding amino acid and to regenerate E-PLP. The catalytic mechanism and structural topology of many aminotransferases,[3] especially L-aspartate aminotransferase (AspAT)[4] and aromatic amino acid aminotransferase(AroAT),[5] have been extensively studied, and their substrate recognition mechanisms have been successfully explained. These enzymes have gained increasing interest due to their high potential for industrial production of amino acids.[6] However, the industrial use of the enzymes has been often hampered by the low equilibrium constants of the reactions that they catalyze.

In the previous study,[7] we isolated a novel amine: pyruvate aminotransferase (APA) from *Vibrio fluvialis*

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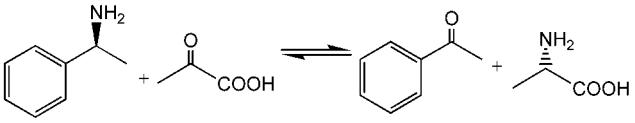
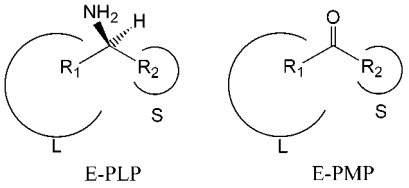
Scheme 1. Reaction Scheme of the Transamination between (*S*)-α-Methylbenzylamine and Pyruvate



Scheme 2. Two-Binding Site Model[a] of E-PLP and E-PMP



[a] L and S denote large and small binding pockets, respectively.

JS17, which showed a unique substrate specificity toward chiral amines containing an aryl group such as α-methylbenzylamine (Scheme 1). The enzyme showed a high enantioselectivity (E >100) for (*S*)-enantiomers of the chiral amines.[7] Unlike other aminotransferase reactions, the equilibrium constant of the reaction that the APA catalyzed was high enough not to limit the reaction, which made the enzyme suitable for production of chiral amines via kinetic resolution as demonstrated elsewhere.[8] Moreover, the APA was capable of deamination of amine and amino acid as well as amination of keto acid, aldehyde, and ketone. Such a broad substrate specificity of the APA rendered various compounds of structural diversity to be used as probes for exploration of the active site structure. Herein, on the basis of the substrate structure−reactivity relationship, an active site model of the APA was developed and empirical rules governing its substrate specificity and stereoselectivity were revealed. The active site model was successful in predicting the reactivity of unknown substrates even without any structural information of the enzyme.

Results

The substrates employed in this study were classified into six groups according to their chemical properties. Amino donors consisted of amines containing aryl group (**A**), amines not containing an aryl group (**B**), and amino acids and their derivatives (**C**). Amino acceptors consisted of keto acids and keto esters (**D**), aldehydes (**E**), and ketones (**F**). Relative reaction rates for amino donor and acceptor were calculated based on those of α-methylbenzylamine (**A1**) and pyruvate (**D1**), respectively. To prevent substrate inhibition,[9] low concentration (5 mM) of the amino donor and acceptor was used except for racemic amino donors (10 mM).

Despite the structural diversity of the substrates, all amino donors and acceptors for the APA could be represented as depicted in Scheme 2. Considering the structural skeleton of the substrates, putative two-binding pockets of different size were assumed to be present at the active site of both E-PLP and E-PMP. For a given substrate, a bulkier side chain was designated as the R_1 group and was assumed to occupy a large binding pocket (L). However, when the substrate contained a carboxylate group, the side chain containing the carboxylate was designated as R_1 irrespective of its size. The rationale of the assumptions will be explained in the Discussion.

Table 1. Amino Donor Specificity of the APA toward Amines Containing Aryl Group

	R_1	R_2	relative reactivity[a] (%)
A1	$-C_6H_5$	$-CH_3$	100 (112.0 [b])
A2			156.5 (161.7)
A3	$-(CH_2)_2C_6H_5$	$-CH_3$	54.2 (61.4)
A4			194.8 (226.9)
A5	$-C_6H_5$	$-CH_2OH$	11.0 (13.3 [c])
A6	$-C_6H_5$-*p*-CH_3	$-CH_3$	35.1 (48.1)
A7	-1-$C_{10}H_7$	$-CH_3$	4.2
A8	$-C_6H_5$-*p*-Br	$-CH_3$	55.2
A9	$-C_6H_5$	$-CH_2CH_3$	19.5
A10	$-C_6H_5$-*p*-NO_2	$-CH_3$	29.3 (31.4)
A11	$-C_6H_5$	-H	115.6
A12	$-CH_2C_6H_5$	-H	23.4
A13	$-(CH_2)_2C_6H_5$	-H	19.7
A14	$-(CH_2)_3C_6H_5$	-H	3.6

[a] The reaction conditions were 10 mM racemic amino donor (**A1**−**A10**), 5 mM pyruvate, and 0.75 U/mL APA at 37 °C. For achiral amines (**A11**−**A14**), concentration of amino donor was 5 mM. [b] Values in parentheses represent relative reactivities of (*S*)-enantiomers (5 mM). [c] In the case of **A5**, (*R*)-enantiomer was used.

Substrate Specificity toward Amino Donor. The most notable feature in the amino donor specificity of the APA is the reactivity toward chiral amines containing an aryl group (from **A1** to **A10** in Table 1). Compared with **A1**, the increase in the size of the R_1 or R_2 group resulted in lower reactivity (**A3**, **A5**−**A10**). Similarly, compared with benzylamine (**A11**) the increase in the size of R_1 of achiral amine bearing two α-hydrogens (**A11**−**A14**) resulted in the decrease in the reactivity. However, cyclization between the R_1 and R_2 group increased the reactivity (**A2**, **A4**). The APA showed high stereoselectivity for (*S*)-enantiomers of the chiral amines (**A1**−**A10**). The reactivity of the (*R*)-enantiomers was lower than 0.1% of that of cognate (*S*)-enantiomers (data not shown). The only exception was α-phenylglycinol (**A5**). Due to the different priority rule in assigning the configuration, (*R*)-**A5** is a fast-reacting enantiomer. It is notable that the racemic amino donors (10 mM, **A1**−**A6** and **A10**) showed lower reactivity than that of the corresponding (*S*)-enantiomers (5 mM, relative reactivity is shown in parentheses in Table 1) although the same amount of the each reactive enantiomer was used in the reaction mixture (also see **B9** and **B17** in Table 2). This result is in accord with the previous one that the nonreactive (*R*)-enantiomers of the amino donors inhibit the APA activity.[10]

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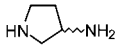
Table 2. Amino Donor Specificity for Amines Not Containing Aryl Group

	R_1	R_2	relative reactivity (%) [a]
B1	-H	-H	0.6
B2	$-CH_3$	-H	0.6
B3	$-CH_2CH_3$	-H	1.7
B4	$-CH_3$	$-CH_3$	3.6
B5	$-(CH_2)_2CH_3$	-H	1.6
B6	$-CH(CH_3)_2$	-H	3.9
B7	$-(CH_2)_3CH_3$	-H	5.6
B8			8.4
B9	$-CH_2CH_3$	$-CH_3$	6.8 (7.1) [b]
B10	$-CH(CH_3)_2$	$-CH_3$	10.1
B11	$-(CH_2)_2CH_3$	$-CH_3$	18.5
B12	$-CH_2CH(CH_3)_2$	$-CH_3$	23.4
B13	$-(CH_2)_4CH_3$	$-CH_3$	42.9
B14	$-(CH_2)_3CH_3$	$-CH_2CH_3$	6.8
B15	$-(CH_2)_3CH(CH_3)_2$	$-CH_3$	24.4
B16			4.5
B17	$-C_6H_{11}$	$-CH_3$	25.9 (29.8)

[a] The reaction conditions were 5 mM amino donor (**B1**−**B8**), 5 mM pyruvate, and 0.75 U/mL APA at 37 °C. For chiral amines (**B9**−**B17**), the concentration of racemic amino donor was 10 mM. [b] Values in parentheses represent relative reactivities of (*S*)-enantiomers (5 mM).

Amino donor reactivities of amines not containing aryl group (**B** group) are presented in Table 2. None of them showed as high reactivity as **A1**. Contrary to the trend observed with the amines in the **A** group, increase in the size of R_1 for a given R_2 moiety (H or CH_3) enhanced the reactivity. Among the amines in the **B** group, 2-aminoheptane (**B13**) displayed the highest reactivity. In addition to the open-chain aliphatic amines, the aliphatic amine whose α-carbon is a member of the ring system showed considerable reactivity (**B8**, **B16**).

The APA showed a strict substrate specificity for amino acids and amino acid derivatives (Table 3). L-Amino acids showing a reactivity of more than 1% of **A1** were L-alanine (**C1**), L-alanine derivatives (**C2**−**C5**), L-serine (**C6**), L-2-aminobutyric acid (**C7**), and 3-aminobutyric acid (**C8**). No D-amino acids and their derivatives showed measurable reactivities. The APA displayed a considerable reactivity toward β-amino acid (**C8**). However, no reactivity was observed for other amino acids bearing non-α amino group such as β-alanine, γ-aminobutyric acid, L-ornithine, and 6-aminohexanoic acid, which are common amino donors for aminotransferases in subgroup II.[11]

Substrate Specificity toward Amino Acceptor. Among keto acids, pyruvate (**D1**) displayed the highest reactivity (Table 4). No reactivity was observed for the keto acids bearing two carboxylate groups (**D2**, **D3**, **D14**).

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Table 3. Amino Donor Specificity for Amino Acids and Amino Acid Derivatives.

	R_1	R_2	relative reactivity[a] (%)
C1	$-CO_2^-$	$-CH_3$	30
C2	$-CONH_2$	$-CH_3$	8.8
C3	$-CO_2CH_3$	$-CH_3$	9.6
C4	$-CO_2C(CH_3)_3$	$-CH_3$	21.8
C5	$-CO_2CH_2C_6H_5$	$-CH_3$	1.2
C6	$-CO_2^-$	$-CH_2OH$	6.4
C7	$-CO_2^-$	$-CH_2CH_3$	5.4
C8	$-CH_2CO_2^-$	$-CH_3$	7.0

[a] The reaction conditions were 5 mM amino donor, 5 mM pyruvate, and 0.75 U/mL APA at 37 °C. For amino acids whose enantiopure forms were not available (3-aminobutyric acid, 3-amino-3-phenylpropionic acid), 10 mM of the racemic form was used. [b] Following the amino acids and amino acid derivatives were inert compounds (relative reactivity of less than 1% of **A1**): D-alanine, D-alaninamide, D-alanine methyl ester, D-alanine benzyl ester, glycine, glycine methyl ester, glycine ethyl ester, glycine *tert*-butyl ester, β-alanine, β-alanine methyl ester, 4-aminobutyric acid, L-ornithine, L-lysine, 6-aminohexanoic acid, L-serine methyl ester, L-serine ethyl ester, D-serine, D-serine methyl ester, D-2-aminobutyric acid, L-valine (**C9**), D-valine, D-valine methyl ester, L-leucine (**C10**), L-leucine *tert*-butyl ester, D-leucine, D-leucine methyl ester, L-aspartic acid (**C11**), L-aspartamide, D-aspartic acid, D-aspartamide, L-glutamic acid (**C12**), L-phenylglycine (**C13**), L-phenylglycine methyl ester, D-phenylglycine (**C14**), D-phenylglycine methyl ester (**C15**), L-phenylalanine (**C16**), L-phenylalninamide, L-phenylalanine methyl ester, D-phenylalanine, D-phenylalninamide, D-phenylalanine methyl ester, 3-amino-3-phenylpropionic acid (**C17**), L-tyrosine, L-tryptophan, L-arginine, D-arginine methyl ester, and D-histidine methyl ester.

Table 4. Amino Acceptor Specificity for Keto Acids and Keto Esters

	R_1	R_2	relative reactivity[a] (%)
D1	$-CO_2^-$	$-CH_3$	100
D2	$-CO_2^-$	$-(CH_2)_2CO_2^-$	0.3
D3	$-CO_2^-$	$-CH_2CO_2^-$	0.2
D4	$-CO_2^-$	$-H$	60.2
D5	$-CO_2^-$	$-CH_2CH_3$	65.1
D6	$-(CH_2)_2CO_2^-$	$-CH_3$	0.5
D7	$-CO_2^-$	$-C_6H_5$	0.2
D8	$-CO_2^-$	$-CH_2C_6H_5$	0.2
D9	$-CO_2^-$	$-C(CH_3)_3$	0.3
D10	$-CO_2^-$	$-CH(CH_3)_2$	0.8
D11	$-CO_2^-$	$-(CH_2)_2CH_3$	0.7
D12	$-CO_2^-$	$-(CH_2)_2C_6H_5$	0.1
D13	$-CO_2^-$	$-(CH_2)_3CH_3$	0.9
D14	$-CO_2^-$	$-CO_2^-$	0.2
D15	$-CO_2CH_3$	$-CH_3$	118.1
D16	$-CO_2CH_2CH_3$	$-CH_3$	131.8
D17	$-CO_2CH_2CH_3$	$-CH(CH_3)_2$	2.5
D18	$-CO_2^-$	$-CH_2OH$	29.2
D19	$-CO_2^-$	$-CH_2SH$	11.0
D20	$-CO_2^-$	$-CH_2F$	55.5

[a] The reaction conditions were 5 mM (*S*)-**A1**, 5 mM amino acceptor, and 0.75 U/mL APA at 37 °C. [b] Following the amino acceptors were inert compounds (relative reactivity of less than 1% of **D1**): ethyl 2-oxophenylbutyrate, dimethyl 2-oxoglutarate, methyl benzoylformate, ethyl benzoylformate.

Compared with **D5**, the keto acids whose R_2 moiety is bulkier than ethyl group showed very low reactivity (**D7**−**D13**).

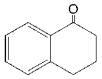
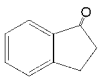
Beside the keto acids, the APA displayed amino acceptor activity toward aldehydes (Table 5). For aldehydes not containing an aryl group (**E1**−**E9**), a further increase in the reactivity was not observed as the size of R_1 became larger than the propyl group (**E3**). Interestingly, as the size of R_1 exceeded the hexyl group, its reactivity decreased dramatically (from **E6** to **E7**). In the case of

Table 5. Amino Acceptor Specificity for Aldehydes

	R_1	R_2	relative reactivity[a] (%)
E1	$-CH_3$	$-H$	15.8
E2	$-CH_2CH_3$	$-H$	36.4
E3	$-(CH_2)_2CH_3$	$-H$	113.5
E4	$-(CH_2)_3CH_3$	$-H$	111.2
E5	$-(CH_2)_4CH_3$	$-H$	106.2
E6	$-(CH_2)_5CH_3$	$-H$	89.9
E7	$-(CH_2)_6CH_3$	$-H$	2.3
E8	$-(CH_2)_7CH_3$	$-H$	0.9
E9	$-(CH_2)_8CH_3$	$-H$	0.4
E10	$-C_6H_5$	$-H$	72.8
E11	$-CH_2C_6H_5$	$-H$	68.5
E12	$-(CH_2)_2C_6H_5$	$-H$	78.3
E13	$-CH{=}CHC_6H_5$	$-H$	31.5

[a] The reaction conditions were 5 mM (*S*)-**A1**, 5 mM amino acceptor, and 0.75 U/mL APA at 37 °C.

Table 6. Amino Acceptor Specificity for Ketones

	R_1	R_2	relative reactivity (%) [a]
F1	$-CH_2CH_3$	$-CH_3$	0.4
F2			6.1
F3	$-(CH_2)_2C_6H_5$	$-CH_3$	3.7
F4			0.9
F5	$-CH(OH)CH_3$	$-CH_3$	4.8
F6	$-CH(CH_3)_2$	$-CH_3$	0.7
F7			0.6
F8	$-C_6H_5$	$-CH_3$	3.1

[a] The reaction conditions were 5 mM (*S*)-**A1**, 5 mM amino acceptor, and 0.75 U/mL APA at 37 °C. [b] Following the amino acceptors were inert comounds (relative reactivity of less than 1% of **D1**): diethyl ketone, 3-heptanone, 4′-methylacetophenone, 4′-bromoacetophenone, propiophenone.

the aldehydes containing an aryl group (**E10**−**E13**), such a steric constraint was not observed. Unlike the high reactivities of the aldehydes, ketones showed relatively low reactivities (Table 6). Among the ketones, the highest reactivity was observed with cyclohexanone (**F2**).

Discussion

The reaction mechanism of aminotransferase has been studied in great detail.[2] Figure 1 shows the proposed reaction mechanism of the APA. Upon binding an amino donor, a Schiff base between an active site lysine and a PLP (internal aldimine) is replaced by a Schiff base between the amino donor and the PLP (external aldimine). The next step is a 1,3-prototropic shift that converts the external aldimine to a ketimine. The active site lysine catalyzes the 1,3-prototropic shift with a defined stereochemistry. Finally, hydrolysis of the ketimine yields PMP and releases a ketone product. Formation of a Michaelis complex between the E-PMP and a different amino acceptor initiates the reverse reaction and eventually regenerates the E-PLP form. Based on the reaction mechanism and the structure of (*S*)-**A1**, a putative two-binding site model was proposed as shown in Figure 1. At the given orientation of the binding pockets, α-hydrogen of (*S*)-**A1** is toward the *si* face at C4′ of the conjugated π-system of the internal aldimine and abstraction of the α-proton occurs on the *si* face, which is observed with most aminotransferases.[2] In the same manner, a proton should be added from the *si* face during the amination step. It means that the carboxyl group of pyruvate should be positioned in the L pocket of E-PMP because the configuration of alanine produced is *S* (i.e., L-alanine). Starting from this preliminary model, we expanded the active site model using the relationship between substrate structure and reactivity to address substrate specificity and stereospecificity of the APA.

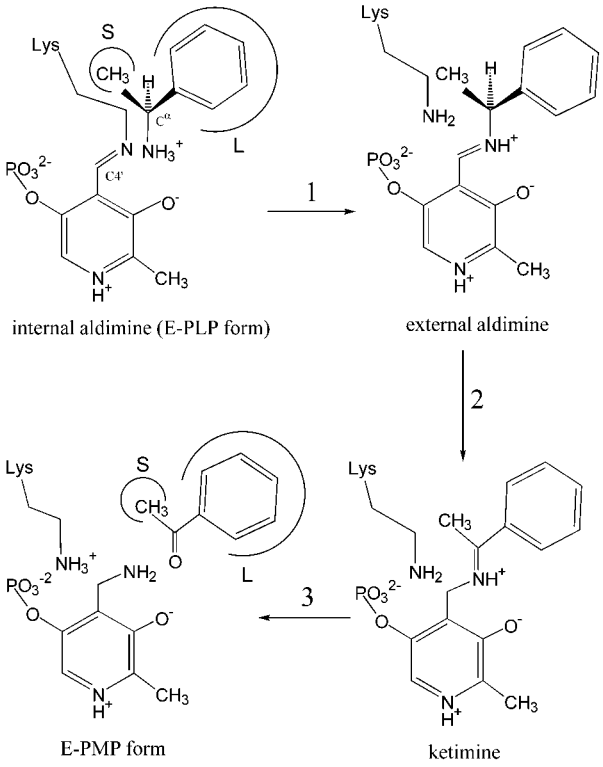


Figure 1. Proposed reaction mechanism of the APA. The PLP cofactor is tentatively positioned with the *re* side at C4′ of the cofactor toward the viewer. Because the C^{α} proton is abstracted on the *si* face[2], L and S pockets are putatively positioned to accommodate the phenyl and methyl group of incoming (*S*)-**A1**, respectively, as shown in the figure. Reaction steps involved are as follows: (1) transaldimination, (2) 1,3-prototropic shift involving proton abstraction from the C^{α} and reprotonation to the C4′, and (3) hydrolysis of ketimine.

To analyze the effect of structural differences in the R_1 and R_2 groups on the activity of the APA, we plotted the relative reactivity against the accessible surface area of the side group for a given counterpart side group (Figures 2−4). As shown in Figure 2, the amino donor reactivity of the amines not containing an aryl group (**B** group) showed a positive correlation with the accessible surface area of the R_1 group irrespective of R_2 group. This result indicates that hydrophobic interaction exists between the L pocket of the E-PLP and R_1 groups. Reactive amino donors showing relative reactivity higher than 50% of **A1** (**A1**, **A2**, **A3**, **A4**, **A8**, and **A11**) exclusively contain an aryl group in R_1 and exhibit higher reactivities than the amines in the **B** group of similar accessible surface area (Figure 2). These results suggest that the hydrophobic interaction in the L pocket with an aryl group is stronger than that with aliphatic group. More evidence comes from the comparison of **A1** and **B17** (see

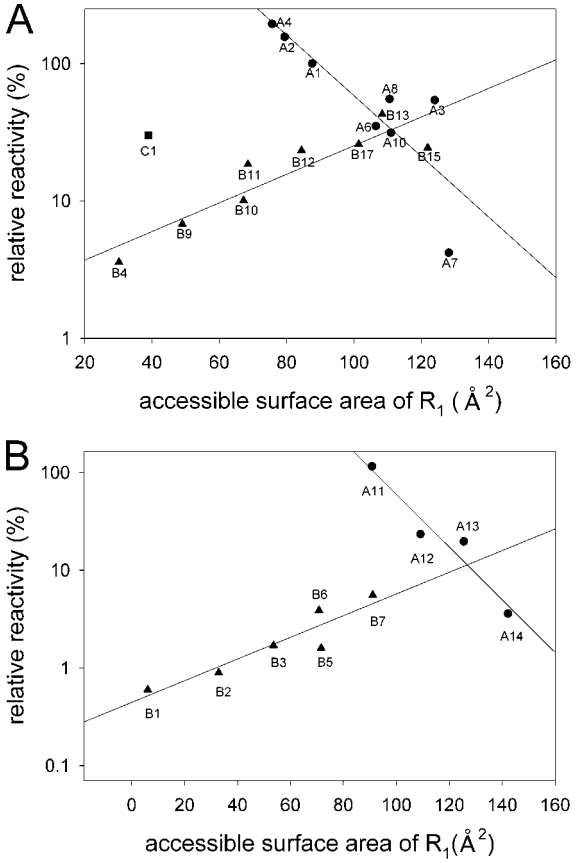


Figure 2. Effect of the size of R_1 for a given R_2 on the amino donor reactivity: (A) $R_2 = CH_3$ and (B) $R_2 = H$. Solid lines represent linear regression of the reactivity of the amines containing an aryl group (●) or the amines not containing an aryl group (▲).

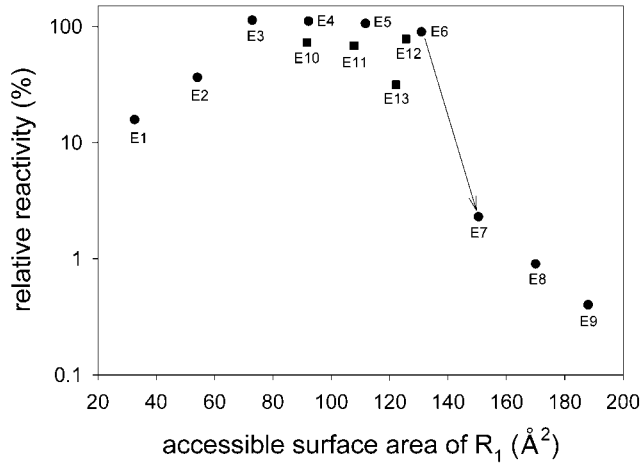


Figure 3. Effect of the size of the R_1 group of aldehyde on the amino acceptor reactivity. ■ and ● represent the aldehydes containing an aryl group and the aldehydes not containing an aryl group, respectively.

Tables 1 and 2). The only difference between the two compounds is the aromatization of R_1 group. However, the reactivity of **A1** is four times higher than that of **B17**. It is notable that the reactivity of the amines containing an aryl group (**A** group) shows a negative correlation with the size of the R_1 group (Figure 2). Thus, the strong hydrophobic interaction with the aryl group appears to be optimized for the phenyl group (**A1**, **A2**, **A4**, and **A11**).

In addition to the hydrophobic recognition mode,

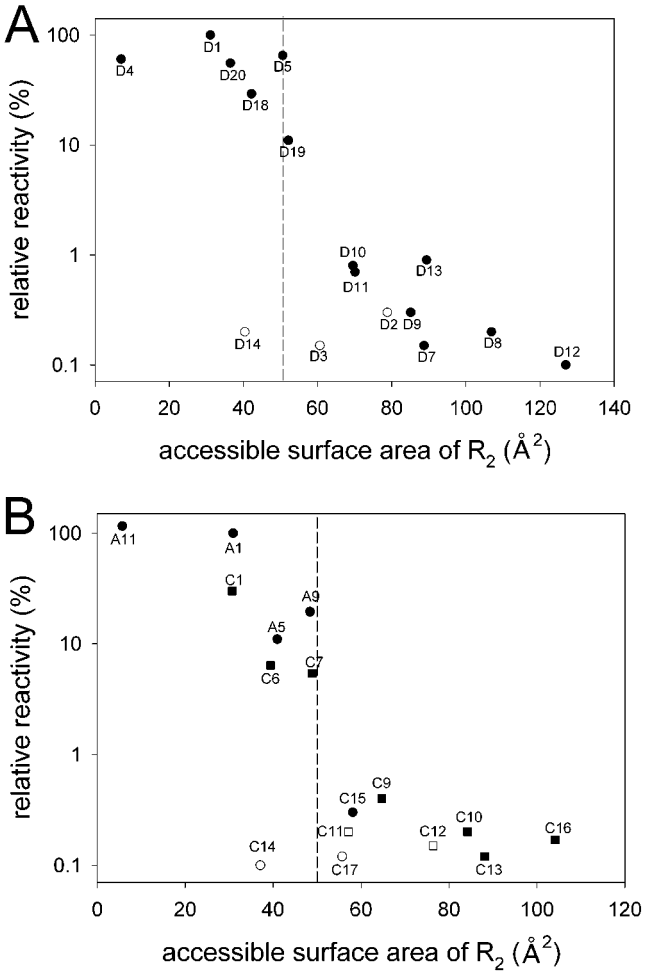


Figure 4. Effect of the size of R_2 for a given R_1 on the reactivity: (A) amino acceptor reactivity when R_1 is carboxylate; (B) amino donor reactivity when R_1 is a phenyl group (●) or carboxylate (■). Open symbols represent the reactivity of substrates whose R_2 contain a carboxylate.

recognition of the acidic group in the L pocket was also observed. Although the carboxylate of **C1** (closed square in Figure 2A) cannot undergo hydrophobic interaction with the L pocket, its reactivity greatly exceeds the value interpolated from the regression line for amines not containing aryl group. This result indicates that the carboxylate of **C1** interacts with the L pocket more strongly than the aliphatic R_1 group of similar accessible surface area. However, four times lower reactivity of **C8** than **C1** indicates that precise spatial positioning of the carboxylate in the L pocket is important for optimal recognition of the acidic group, which appears to be evolved for the recognition of pyruvate. It is notable that removal of the negative charge of **C1** by modification resulted in lower reactivity (**C2**−**C5** in Table 3), suggesting that electrostatic interaction is important for the recognition of the acidic group. In contrast, in the case of amino acceptor, the removal of negative charge increases the reactivity (compare **D1**, **D15**, and **D16** in Table 4). The dual recognition mode for hydrophobic and acidic group is commonly observed with the side-chain pockets of AroAT and AspAT.[12]

The L pocket of E-PMP showed different properties from that of E-PLP (Figure 3). First, aldehydes contain-

(12) Nobe, Y.; Kawaguchi, S.; Ura, H.; Nakai, T.; Hirotsu, K.; Kato, R.; Kuramitzu, S. *J. Biol. Chem.* **1998**, *273*, 29554−29564.

ing an aryl group (**E10**−**E13**) showed similar reactivities and the steric hindrance of R_1 group larger than phenyl group, which was observed with the L pocket of E-PLP, was not observed. Second, no correlation was observed between the size of aliphatic R_1 groups (● symbols in Figure 3) and their reactivity. Third, the reactivity of the aliphatic aldehydes (**E4**−**E6**) was higher than that of the aldehydes containing an aryl group with a similar size (**E10**−**E13**). These results suggest that hydrophobic interaction in the L pocket of E-PMP is not as important as that of E-PLP. It is noteworthy that the amino acceptor reactivity of aldehyde dramatically decreases as the carbon number of R_1 group exceeds six (denoted by arrow in Figure 3). Therefore, the L pocket of E-PMP appears to accommodate the R_1 moiety up to a hexyl group, and further increase in the R_1 size appears to cause severe steric hindrance.

As shown in Table 6, no ketone showed amino acceptor reactivity higher than 10% of **D1**. The low reactivity of the ketones appears to stem from relatively low electrophilicity of the carbonyl carbon compared with α-keto acid and aldehyde. We have other evidence supporting the importance of the electrophilicity of the carbonyl carbon for amino acceptor reactivity. Ester forms of **D1** (i.e., **D15** and **D16**) are more reactive than **D1** presumably due to the higher electrophilicity of the carbonyl carbon. Similarly, **E13** showed lower reactivity than **E12** due to increased conjugation of π-bonds and consequent decrease in the electrophilicity. Therefore, the reactivity of amino acceptor appears to be determined by the degree of susceptibility of the carbonyl carbon to nucleophilic attack by the amino group of PMP rather than the degree of hydrophobic interaction between the R_1 group and the L pocket.

The most important property of the S pocket is a steric constraint. In contrast to the relatively spacious room in the L pocket, the S pockets of both E-PLP and E-PMP appear to accommodate R_2 group of accessible surface area up to approximately 50 Å^2 that is comparable to ethyl group (denoted by dashed line in Figure 4). Another important property of the S pocket is a strong repulsion for an acidic group. As shown in Figure 4 (open symbols), no amino donors and acceptors containing a carboxylate in R_2 showed reactivity higher than 1% of **A1** and **D1**, respectively. In the case of **D14**, both R_1 and R_2 are carboxylates. Although the carboxylate is not expected to undergo steric hindrance in the S pocket, **D14** is inert due to the electrostatic repulsion. Similarly, oxalacetate (**D3**) and α-ketoglutarate (**D2**) are inert although they are common amino acceptors for other aminotransferases.[2] The electrostatic repulsion makes the carboxylate of α-keto acids (**D1**, **D5**, **D18**, **D19**, and **D20**) occupy the L pocket, which exclusively yields L-amino acids after amination.[13]

In the previous study,[10] we found that the (*R*)-enantiomers of chiral amines (**A1**-**A4**) form Michaelis complexes with E-PLP with high affinity and inhibit enzyme activity severely. It is likely that the APA has been evolved to control the stereoselectivity for amino donor after the formation of Michaelis complex. For instance, due to the steric constraint in the S pocket, phenyl and methyl group of (*R*)-**A1** occupy the L and S pocket, respectively, as those of (*S*)-**A1** do. However, relative positions of amino group and α-hydrogen are opposite in the two Michaelis complexes. Therefore, the Michaelis complex between the E-PLP and (*R*)-**A1** cannot undergo further reaction although (*R*)-**A1** shows high affinity toward the E-PLP.

In conclusion, the active site model proposed herein is successful in explaining the observed substrate specificity and stereoselectivity of the APA in both amination and deamination steps and in predicting the reactivity of an unknown substrate. However, it is obvious that the active site model should be verified by a precise three-dimensional structure of the enzyme. Then, the residues associated with the dual recognition mechanism in the L pocket and the electrostatic repulsion in the S pocket will be clarified.

Experimental Section

Chemicals. Most of the chemicals used in this work were of the highest grade available and purchased from Aldrich Chemical Co. and Sigma Chemical Co. (*R*)- and (*S*)-enantiomers of **A2**, **A3**, and **A4** were from Chirotech Technology Ltd. Amino acids and their derivatives that are not available from Aldrich and Sigma Chemical Co. were purchased from Bachem Chemical Co.

Enzyme Assay. All enzyme assays were carried out at 37 °C, and the enzyme concentration was 0.75 U/mL. One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmol of acetophenone in 1 min at 50 mM of (*S*)-**A1** and 10 mM of **D1**. Typical reaction volume was 200 μL. Both concentrations of amino donor and acceptor in the reaction mixture were 5 mM except racemic amino donor (10 mM). For the measurement of amino donor reactivity, **D1** was used as an amino acceptor, whereas **D5** was used for L- or D-alanine and their derivatives. For the measurement of amino acceptor reactivity, (*S*)-**A1** was used as an amino donor except that **A11** was used for **F8**. The reaction was allowed to proceed for 10 min and then was stopped by addition of 75 μL of 16% (v/v) perchloric acid. After the reaction, residual pyruvate or produced acetophenone was analyzed by HPLC for measurements of the initial reaction rates.

Analytical Methods. Chiral analysis of alanine was carried out by using a Crownpak CR (Daicel Co., Japan) column with a Waters HPLC system (Milford, MA) as described elsewhere.[14] Analysis of acetophenone was performed with a Symmetry HPLC column (Waters, USA) with isocratic elution of acetonitrile/water (50/50 v/v) at 1 mL/min. Pyruvate and α-ketobutyric acid were analyzed with an Aminex HPX-87H column (Bio-Rad, USA). H_2SO_4 (5 mM) aqueous solution was used as an eluent at a flow rate of 0.6 mL/min. All UV detections were carried out at 205 nm.

Accessible Surface Area Measurement. Measurement of accessible surface areas of the substrates was carried out with Sybyl 6.5 program as follows. The initial structures of the substrates were energy-minimized using conjugate gradient minimization until the maximum derivatives was less than 0.001 kcal/mol/Å. In all minimizations, a cutoff distance of 11 Å was used with CVFF force field. With the energy-minimized conformers of the substrates, solvent-accessible surface areas of R_1 and R_2 groups were calculated using Connolly's method[15] with a probe size of 1.4 Å.

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(13) The enantiomeric excesses of the L-amino acids produced after amination of **D1**, **D5**, **D18**, **D19**, and **D20** were all above 99%.

(14) Shin, J.-S.; Kim, B.-G. *Biotechnol. Bioeng.* **1999**, *65*, 206−211.
(15) Connolly, M. L. *Science* **1983**, *221*, 709−715.