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Mutation of Arginine 98, Which Serves as a Substrate-Recognition Site of D-Amino Acid Aminotransferase, Can Be Partly Compensated for by Mutation of Tyrosine 88 to an Arginyl Residue[1]

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D-Amino acid aminotransferase is the only aminotransferase that catalyzes the transamination of D-amino acids. We studied the role of the binding site for the α-carboxyl group of substrates, which is presumably crucial for the unique stereospecificity of the enzyme. The site-directed mutagenesis of Arg98, which is the putative carboxyl-binding site, as judged on the basis of X-ray crystallographic studies [Sugio, S., Petsko, G.A., Manning, J.M., Soda, K., and Ringe, D. (1995) *Biochemistry* 34, 9661-9669], by replacement with methionine and lysine, resulted in decreases in the k_{max} values and increases in the K_d values for both amino donors and amino acceptors. The introduction of another mutation, that of Tyr88, which is located near Arg98 in the spacial structure, by replacement with arginine, in addition to the above Arg98 mutation, resulted in increases in the k_{max} values but little change in the K_d values. These results suggest that Arg98 constitutes the carboxyl-binding site for the substrate, efficient catalysis by the enzyme being facilitated upon binding. The mutant enzymes are also relieved from inhibition by high concentrations of α-ketoglutarate, which is an inherent character of the wild-type enzyme. Therefore, Arg98 is also responsible for the inhibition by α-ketoglutarate.

Key words: D-amino acid aminotransferase, carboxyl-binding site for substrates, inhibition by α-ketoglutarate, site-directed mutagenesis.

Bacterial D-amino acid aminotransferase (D-AAT) requires pyridoxal 5′-phosphate (PLP) as a cofactor, and participates in the metabolism of D-amino acids, some of which are indispensable for bacteria as components of the peptidoglycan layer of cell walls. Thus, the enzyme has been regarded as a target for the development of antibacterial agents serving, for example, as suicide substrates (*1-3*).

We purified a thermostable D-AAT from an isolated thermophile, *Bacillus* sp. YM-1 (*4*), and cloned and sequenced its gene (*5*). To elucidate reaction mechanism of the enzyme in detail, we have studied the functions of an active-site lysyl residue (Lys145; *6-9*), two seryl residues (Ser146 and Ser180; *10, 11*), three cysteinyl residues (Cys142, Cys164, and Cys212; *10*), a threonyl residue (Thr31; *11*), a tryptophanyl residue (Trp139; *12, 13*), and a leucyl residue (Leu201; *14*) by means of site-directed mutagenesis. The three-dimensional structure of D-AAT in the pyridoxamine 5′-phosphate (PMP) form has been determined crystallographically (*15*).

D-AAT of *Bacillus* sp. YM-1 and branched-chain L-amino acid aminotransferase (BCAT; *16*) of *Escherichia coli* show significant similarity in their primary structures (28%). They belong to the same family of proteins, which is clearly distinct from other families of proteins including all other aminotransferases (*17, 18*). An X-ray crystallographic study of hexameric *E. coli* BCAT has demonstrated that the folding of the dimer unit is very similar to that of D-AAT (*19*).

During their reactions, D-AAT and BCAT catalyze the hydrogen transfer between C-4′ of the cofactor and C-2 of the substrate stereospecifically on the *re*-face of the coenzyme-substrate Schiff base intermediate, in contrast to other aminotransferases catalyzing *si*-face specific hydrogen transfer (*20*). In PLP-dependent enzyme reactions, the bond to be broken is situated perpendicularly as to the plane of the substrate-cofactor imine (*21*). Thus, the Cα-H bond of each amino donor, which is broken upon transamination, is located perpendicularly on the *re*-face of the plane in the active sites of D-AAT and BCAT (Fig. 1). The difference in the substrate stereospecificities of these enzymes suggests that the orientations of substrates bound to their active site are opposed. The binding sites for the α-carboxyl groups of substrates of D-AAT and BCAT are probably situated at positions opposite to each other with respect to the C1-C4 axis of the cofactor portion of the external Schiff base. Therefore, we have studied the

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Abbreviations: AspAT, aspartate aminotransferase; BCAT, branched chain L-amino acid aminotransferase; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; D-AAT, D-amino acid aminotransferase; MES, 2-(*N*-morpholino)ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane.

binding site for the α-carboxyl groups of substrates of D-AAT.

Site-directed mutagenesis studies on D-AAT showed that Lys145, which is bound with PLP through an aldimine linkage at the active center, acts as the base for abstracting α-hydrogen from a substrate amino acid (*6-9*). X-Ray crystallographic studies on the enzyme (*15*) showed that Arg98 is located near Lys145, probably acting as the carboxyl-binding site (Fig. 2). We have constructed a mutant enzyme, R98M, and characterized it, particularly its binding affinity for substrates. Tyr88 is situated near Arg98, the distance between NE of Arg98 and CE2 of Tyr88 being approximately 3.7 Å (Fig. 2). The side chain of Tyr88 undergoes no hydrogen bonding with other functional groups around it, and thus one can expect that Tyr88 can be replaced by other residues without deforming the

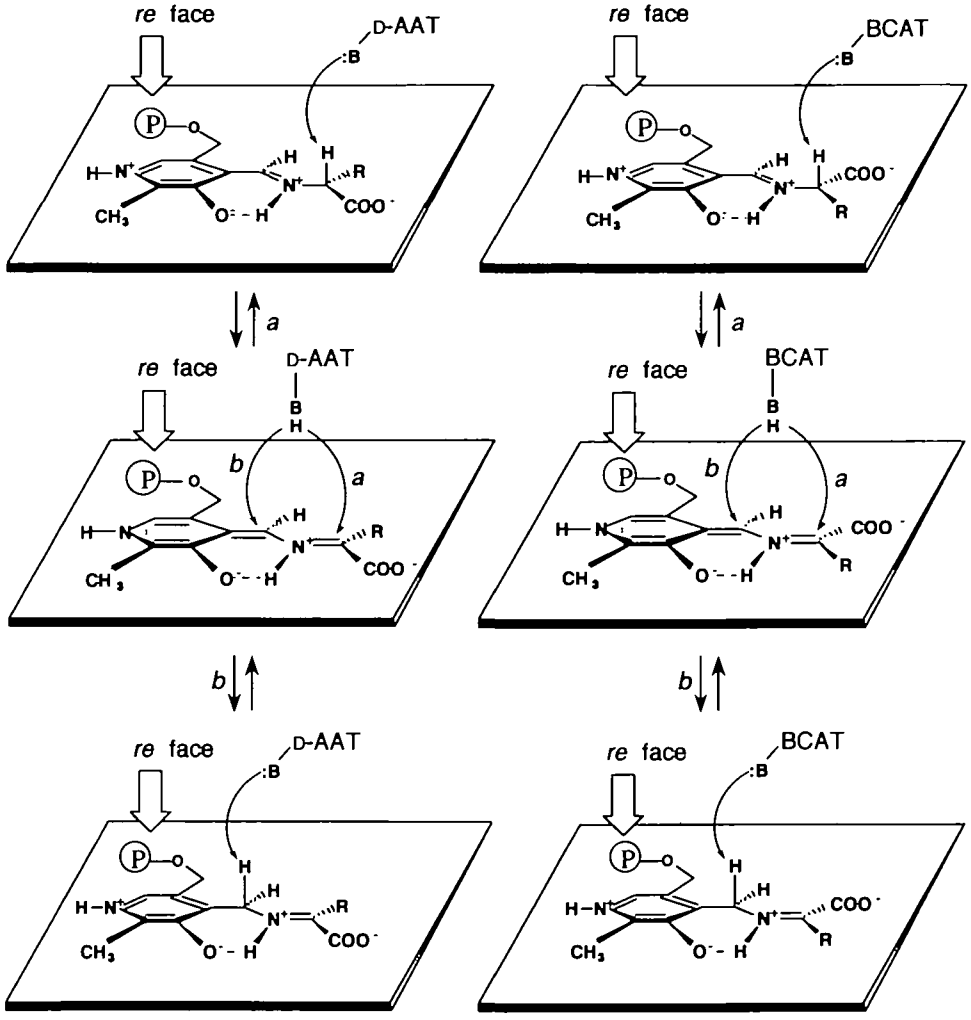

Fig. 1. ***re*-Face specific hydrogen transfer catalyzed by D-AAT and BCAT.** B indicates the basic side chain of the enzymes serving as a base for abstracting and transferring hydrogen: Lys145 of D-AAT (*6-9*) and Lys159 of BCAT (*19*). The hydrogen attached to the base is transferred to either (a) C-2 of the substrate or (b) C-4′ of the cofactor.

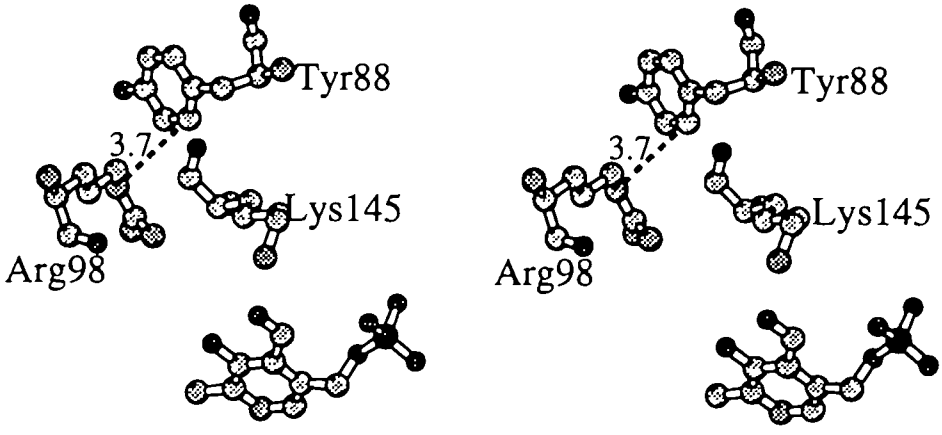

Fig. 2. **Stereo diagram showing the positions of Tyr88, Arg 98, and Lys145 with respect to PLP at the active site of D-AAT (*15*).** The numbers indicate the distance (Å) between NE of Arg98 and CE2 of Tyr88, shown by dashed lines.

enzyme. In particular, arginine introduced in place of Tyr88 of the R98M mutant enzyme possibly has a similar function to Arg98 in the wild-type enzyme, and therefore the carboxyl-binding site lost with the R98M mutation will be recovered in the double mutation enzyme. We present here some evidence suggesting that Arg98 serves as the binding site for the α-carboxyl groups of substrates to maintain the efficient catalytic function of the enzyme upon proper binding of the substrate.

MATERIALS AND METHODS

Materials—Plasmid pAZZI, which was derived from pEMBL18 and contains the D-AAT gene, was prepared as described previously (*10*). The oligonucleotides used for mutagenesis were synthesized by means of phosphoramidite chemistry. All other reagents and chemicals were of analytical grade.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out as described previously (*10, 14*) with the following three oligonucleotides as primers, 5′-TTGATGCGCTTTAGGCGAAGT-3′ for the R98K mutant enzyme, 5′-GAATTGATGCGCCATAGGCGAAGTTCC-3′ for the R98M mutant enzyme, and 5′-AACTTGAAAACGAATATGCCC-3′ for the Y88R mutant enzyme. The mutagenized nucleotides are underlined.

Purification of the Enzymes—Mutant enzymes were purified from *E. coli* JM109 cells transformed with pAZZI encoding the gene of each mutant enzyme as described previously (*14, 22*). The pyridoxamine phosphate (PMP) forms of the wild-type and mutant enzymes were prepared by incubation of each enzyme with 600 mM D-alanine in 20 mM potassium phosphate buffer (pH 7.3) at room temperature for 10 min, followed by filtration through Sephadex G-25.

Spectrophotometric Measurements—Absorption spectra were obtained with a Shimadzu UV260 spectrophotometer. Circular dichroism was measured with a Jasco J-600 spectrophotometer at 25°C, with a 1-cm light path cell.

Steady-State Kinetic Study—The rate of the overall reaction was determined by measuring the time-dependent decrease in the absorbance at 340 nm with a Shimadzu MPS 2000 spectrophotometer as described previously, except that the following MBC buffer was used instead of Tris-HCl buffer (*14*). The reaction mixture (1 ml) for the steady-state kinetic studies comprised MBC buffer consisting of: 100 mM (final concentration) MES, BICINE, CAPS, and NaCl, 50 μM PLP, 0.2 mM NADH, appropriate amounts of the wild-type or mutant D-AAT, various concentrations of amino acid and keto acid as substrates, and 10 units/ml of a suitable coupling enzyme. When D-alanine and α-ketoglutarate were used as substrates, lactate dehydrogenase was used as a coupling enzyme. Malate dehydrogenase was used for D-aspartate/pyruvate substrate pairs. The reaction was started by the addition of an α-keto acid. The kinetic parameters for D-alanine and α-ketoglutarate were determined with 10 mM α-ketoglutarate and 100 mM D-alanine, respectively. Plots of the initial rates against the concentrations of substrates were fitted by nonlinear regression to a rectangular hyperbolic curve given by the equation,

$$V = V_{max}[S]/(K_m^{app} + [S]) \quad (1)$$

where [S] is the concentration of substrate and K_m^{app} is the apparent Michaelis constant. Data fitting was carried out with KaleidaGraph software (Abelbeck Software, USA).

Pre-Steady State Kinetic Analysis of Half Reactions—The half reactions catalyzed by the PLP and PMP forms of the enzymes were assayed at 37°C with D-alanine and pyruvate as substrates, respectively, in 100 mM MBC buffer (pH 7.7) with a SX-18 Stopped-flow Reaction Analyser equipped with a Data-Processing Unit for curve fitting (Applied Photophysics, England). The enzyme concentration in the reaction mixture was about 5 μM, and the substrate concentration varied from 0 to 400 mM.

RESULTS

Purification and Characterization of the Wild-Type, R98K, R98M, and Y88R/R98M Mutant Enzymes—The wild-type and mutant enzymes were purified by DEAE-Toyopearl column chromatography to homogeneity, as judged on SDS-polyacrylamide gel electrophoresis. The amount of each enzyme purified from 4 g (wet weight) of the transformant cells was about 30 mg as protein. The circular dichroism spectra of the wild-type and mutant enzymes in the far-UV region were basically identical. This suggests that the mutant enzymes have secondary structures similar to that of the wild-type enzyme. We determined the PLP contents of the wild-type and mutant enzymes by Adams' fluorometric method (*23*). The contents of the wild-type (1.0 mol/mol subunit) and R98K mutant (1.0 mol/mol) enzymes were slightly higher than those of the R98M (0.89 mol/mol) and Y88R/R98M (0.80 mol/mol) mutant enzymes. These results suggest that the presence of a basic side-chain at position 98 is required for the tight binding of the enzyme with PLP. The three-dimensional structure of D-AAT revealed that Arg98 is not situated near the 5′-phosphate or 3′-hydroxyl group of the coenzyme (*15*). However, the positive charge at position 98 probably acts to stabilize the coenzyme binding through an indirect interaction with its anionic group(s). The wild-type and mutant enzymes showed similar UV-visible spectra, with absorption maxima at 280, 333, and 415 nm. The ratios of the absorbance at 333 and 415 nm relative to the 280-nm absorbance of the wild-type and mutant enzymes were: wild-type enzyme, 0.082 and 0.22; R98K, 0.046 and 0.22; R98M, 0.048 and 0.20; and Y88R/R98M, 0.063 and 0.20, respectively. The absorbance ratios of D-AAT, in particular the ratio, A_{333}/A_{280}, are readily modified under various conditions, and various mutant enzymes show different values (*10, 12, 14*). The specific activities of the purified R98K, R98M, and Y88R/R98M mutant enzymes were 0.92, 0.036, and 0.59% of that of the wild-type enzyme, respectively. These results indicate that Arg98 of D-AAT plays an important role in catalysis.

Kinetic Parameters for Overall Reactions—The apparent kinetic parameters for the overall reactions with D-alanine and α-ketoglutarate catalyzed by the wild-type and mutant enzymes are shown in Table I. High concentrations of α-ketoglutarate inhibit the overall reaction catalyzed by the wild-type enzyme, as described below. Therefore, the kinetic parameters for D-alanine were obtained with α-ketoglutarate at concentrations of less than 10 mM. The V_{max}/K_m^{app} values of the R98K mutant enzyme for D-alanine and α-ketoglutarate were 0.31 and 0.46% of those of the

TABLE I. **Kinetic parameters for the overall transamination.**[a]

	V_{max} (μmol·min^{-1}·mg^{-1})		K_m^{app} (mM)		V_{max}/K_m^{app} (μmol·min^{-1}·mg^{-1}·M^{-1})	
	D-Alanine	α-KG[b]	D-Alanine	α-KG[b]	D-Alanine	α-KG[b]
Wild-type	230 (±0.73%)	350 (±1.7%)	6.0 (±3.0%)	5.1 (±7.4%)	39,000 (±2.4%)	68,000 (±6.2%)
R98K	3.2 (±2.2%)	6.2 (±2.6%)	27 (±6.3%)	20 (±8.0%)	120 (±4.0%)	310 (±5.5%)
R98M	0.082 (±2.9%)	1.0 (±1.6%)	6.2 (±13%)	51 (±3.3%)	13 (±12%)	20 (±1.9%)
Y88R/R98M	1.3 (±5.0%)	6.4 (±3.6%)	4.6 (±12%)	30 (±10%)	270 (±7.0%)	220 (±6.8%)

[a]Standard errors are shown in parentheses. [b]α-KG denotes α-ketoglutarate. The values were determined with Eq. 1 (see text) at α-KG concentrations lower than 50 mM, because high concentrations of α-KG inhibit the enzyme, as shown in the text.

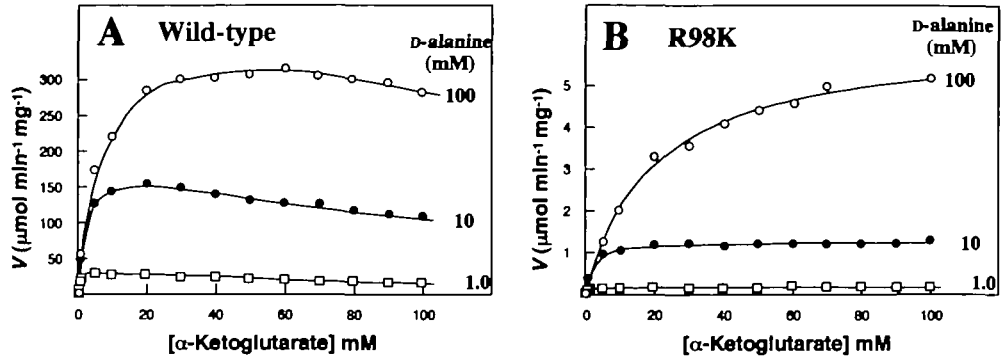

Fig. 3. **Dependence of the rates of the overall reactions catalyzed by the wild-type and mutant enzymes on the α-ketoglutarate concentration.** The reaction mixture (1 ml) comprised 100 mM MBC buffer (pH 7.7), 100 (○), 10 (●), or 1.0 (□) mM D-Ala, 50 μM PLP, 0.2 mM NADH, 10 units of lactate dehydrogenase, α-ketoglutarate as indicated, and 0.65 μg of the wild-type enzyme (A), 4.2 μg of the R98K mutant enzyme (B), 65 μg of the R98M mutant enzyme (C), or 10 μg of the Y88R/R98M mutant enzyme (D). The reaction was started by the addition of α-ketoglutarate and then the decrease in absorbance at 340 nm was monitored at 37°C.

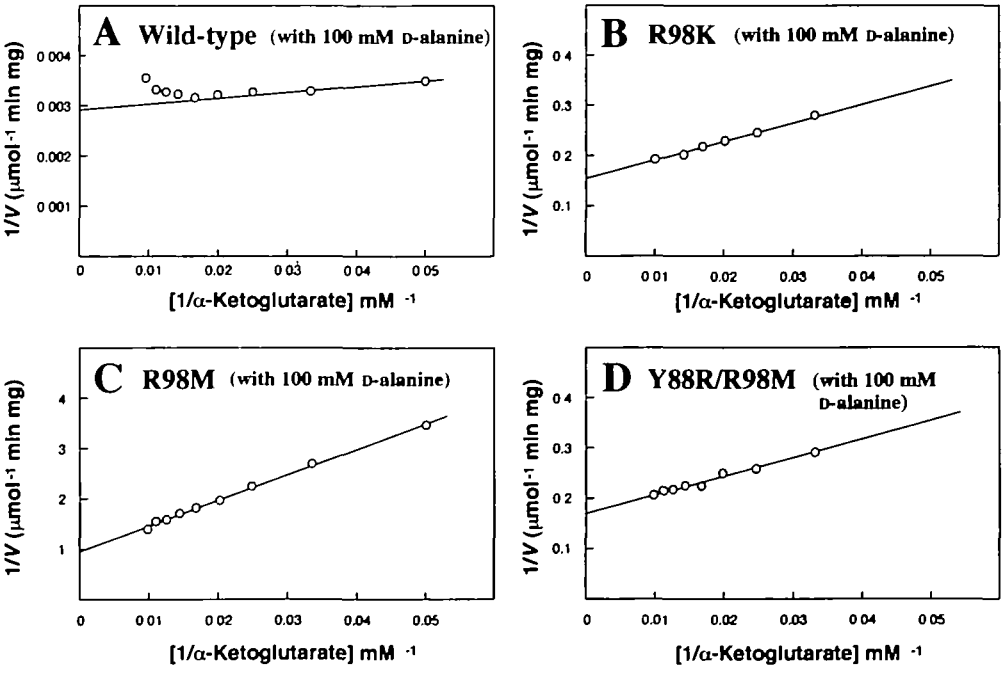

Fig. 4. **Double reciprocal plots of the rates of the overall reactions catalyzed by the wild-type and mutant enzymes against the concentration of α-ketoglutarate.** D-Alanine (100 mM) was used as the amino donor. Other conditions were the same as those given in Fig. 3.

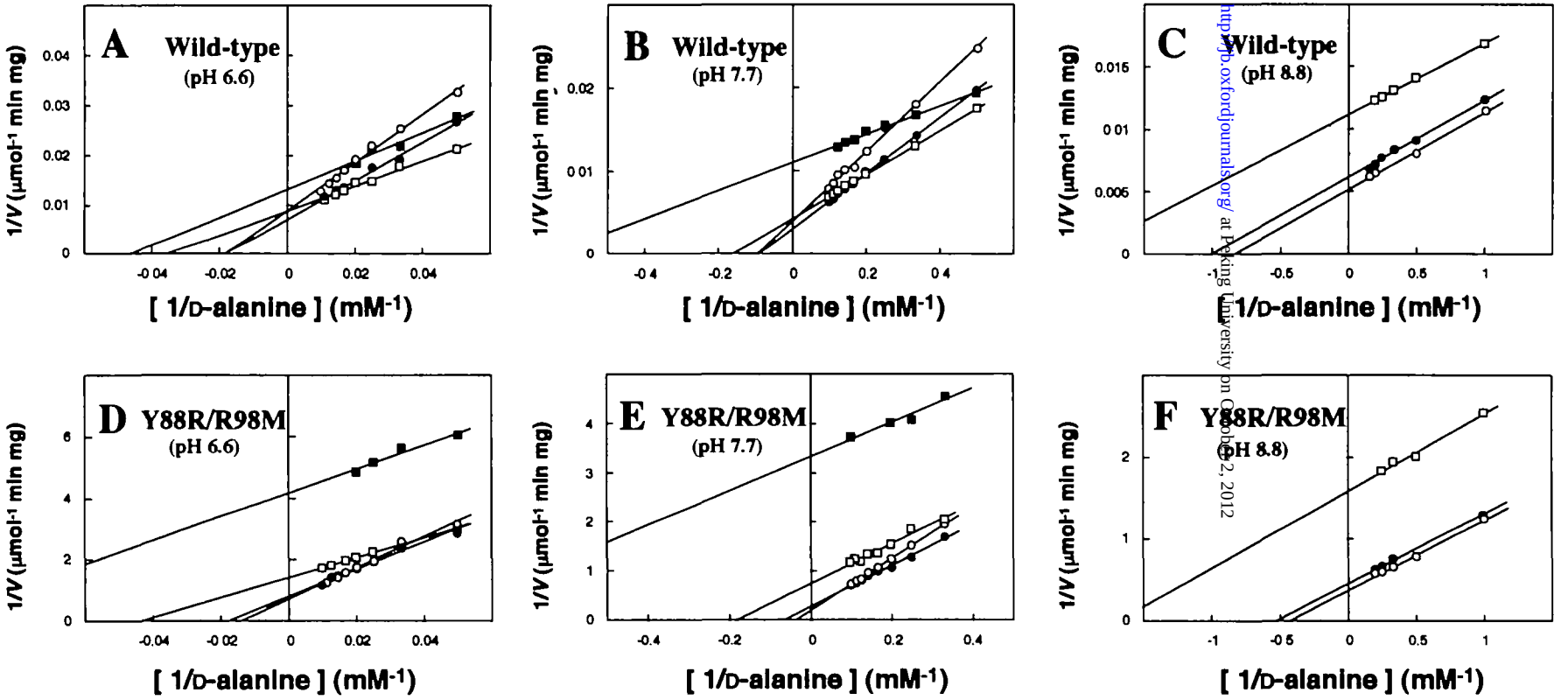

Fig. 5. **pH-dependence of the effects of the concentrations of D-alanine and α-ketoglutarate on the initial velocities of the overall reactions catalyzed by the wild-type and mutant enzymes.** Double reciprocal plots of velocity against the D-alanine concentration with several fixed concentrations of α-ketoglutarate were obtained at various pHs. The reaction mixture comprised 100 mM MBC buffer (pH 6.6 for A and D, pH 7.7 for B and E, and pH 8.8 for C and F), D-alanine as indicated, 100 (○), 50 (●), 10 (□), or 2.0 (■) mM α-ketoglutarate, and 0.65 μg of the wild-type (A, B, C), or 10 μg of the Y88R/R98M mutant (D, E, F) enzyme. Other conditions were the same as those given in Fig. 3.

wild-type enzyme, respectively. The mutation of Arg98 to Met lowered the V_{max}/K_m^{app} values by more than four orders of magnitude. However, the additional mutation of Tyr88 to Arg in the R98M mutant enzyme increased the V_{max}/K_m^{app} values for D-alanine and α-ketoglutarate about 20- and 10-fold, respectively. These results indicate that the function of Arg98 lost with the mutation can be partially compensated for by the arginine introduced in place of Tyr88.

Inhibitory Effect of α-Keto Acids on the Overall Reaction Catalyzed by the Wild-Type Enzyme—The rate of the overall reaction catalyzed by the wild-type enzyme was measured with various concentrations of α-ketoglutarate (1–100 mM) and fixed concentrations of D-alanine at pH 7.7. The reaction rates became maximum at about 5, 20, and 60 mM α-ketoglutarate with 1, 10, and 100 mM D-alanine, respectively, and then decreased with further increases in the concentration of α-ketoglutarate (Fig. 3A). A Lineweaver-Burk plot of the rates against the concentrations of α-ketoglutarate suggested that α-ketoglutarate at high concentrations inhibits the overall transamination catalyzed by the wild-type enzyme (Fig. 4A). Pyruvate showed a similar inhibitory effect at high concentrations when used as the amino acceptor with D-aspartate as the amino donor (data not shown). However, the V_{max}/K_m^{app} values for α-ketoglutarate were not influenced by the concentration (between 1 and 400 mM) of D-alanine (data not shown). This indicates that the wild-type enzyme is not inhibited by D-alanine, at least in this concentration range.

Inhibitory Effect of α-Ketoglutarate on the Overall Reactions Catalyzed by the R98K, R98M, and Y88R/R98M Mutant Enzymes—The rates of the overall reactions catalyzed by the mutant enzymes, R98K, R98M, and Y88R/R98M, with fixed concentrations of D-alanine increased with increases in the concentration of α-ketoglutarate. Double-reciprocal plots of the initial velocity of the reactions against the concentration of α-ketoglutarate with fixed concentrations of D-alanine gave straight lines (Fig. 4). Therefore, the mutant enzymes are distinct from the wild-type enzyme in that they are not inhibited by high concentrations of α-ketoglutarate: therefore Arg98 causes the inhibition by α-ketoglutarate.

Effect of pH on the Inhibitory Effect of α-Ketoglutarate—We studied the pH-dependence of the inhibitory effect of α-ketoglutarate on the overall reactions catalyzed by the wild-type enzyme (Fig. 5, A, B, and C). Double-reciprocal plots of the initial velocity against the concentration of D-alanine in the presence of several fixed concentrations of α-ketoglutarate did not give parallel lines at pH 6.6 and 7.7 (Fig. 5). This suggests that the reaction does not proceed through a "ping-pong bi-bi" mechanism, clearly in contrast with the reactions catalyzed by other aminotransferases (*24*). On the other hand, parallel lines were obtained at pH 8.8; the overall reaction proceeds through a ping-pong bi-bi mechanism at this pH value. These results indicate that the positive charge of the Arg98 residue is responsible for the inhibition by α-ketoglutarate. Similar results were obtained with the Y88R/R98M mutant enzyme, but the inhibition by α-ketoglutarate was much weaker than that for the wild-type enzyme (Fig. 5, D, E, and F).

pH Profiles of Kinetic Parameters for D-Alanine—pH profiles of the log V_{max}, log V_{max}/K_m^{app}, and log $1/K_m^{app}$ values for D-alanine in the overall reactions catalyzed by the wild-type enzyme, and the R98K and Y88R/R98M mutant enzymes were obtained with 10 mM α-ketoglutarate (Fig. 6). The wild-type enzyme was similar to the Y88R/R98M mutant enzyme, but the R98K mutant enzyme was distinct from the others in the pH profiles of the log $1/K_m^{app}$ and log V_{max}/K_m^{app} values. The discrepancy is probably due to the pH-dependence of the inhibition by α-ketoglutarate described above.

Pre-Steady State Kinetic Analysis of Half Reactions—Because of the inhibition by α-keto acids, the K_m^{app} and V_{max} values obtained for the overall reactions do not simply indicate the affinity of the enzyme for a substrate and the catalytic efficiency of the enzyme, respectively. However, the overall reaction is composed of two half reactions, which can be interpreted straight-forwardly. Therefore, we analyzed the pre-steady state kinetics of the half reactions.

The PLP forms of the wild-type and mutant enzymes exhibited absorbance at 415 nm. Upon the addition of an amino donor, the 415-nm band shifted rapidly to 333 nm,

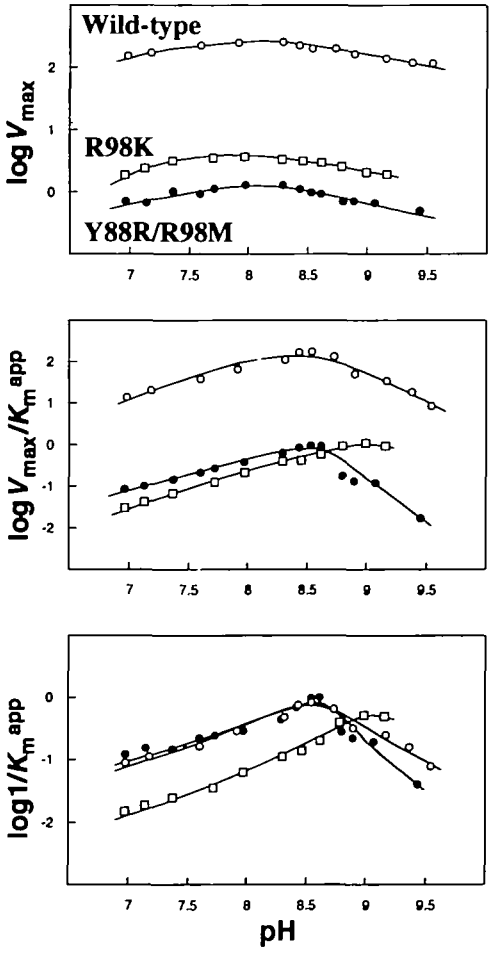

Fig. 6. **pH dependence of the V_{max}, V_{max}/K_m^{app}, and $1/K_m^{app}$ values for D-alanine in the reactions catalyzed by the wild-type enzyme, and the R98K and Y88R/R98M mutant enzymes.** The reaction mixture comprised 100 mM MBC buffer at the indicated pH, 10 mM α-ketoglutarate, and 0.65 μg of the wild-type enzyme (○), or 4.2 μg of the R98K (□), or 10 μg of the Y88R/R98M (●) mutant enzyme. Other conditions were the same as those given in the legend of Fig. 3.

TABLE II. **Kinetic parameters for the half reactions.**[a]

Enzyme	k_{max} (s^{-1})		K_d (mM)		k_{max}/K_d ($M^{-1} \cdot s^{-1}$)	
	D-Alanine	Pyruvate	D-Alanine	Pyruvate	D-Alanine	Pyruvate
Wild-type	510 (±7.8%)	570 (±8.1%)	12 (±12%)	0.87 (±11%)	43,000 (±4.4%)	650,000 (±4.2%)
R98K	17 (±14%)	67 (±13%)	200 (±19%)	88 (±22%)	87 (±5.5%)	770 (±9.2%)
R98M	n.d.[b]	75 (±19%)	n.d.[b]	190 (±24%)	14[c] (±1.5%)	400 (±6.8%)
Y88R/R98M	100 (±8.2%)	140 (±7.1%)	370 (±1.2%)	130 (±11%)	130 (±0.40%)	1,100 (±3.5%)

[a]Standard errors are shown in parentheses. [b]The values could not be determined under the conditions used. [c]$k_{app}/[S]$, which was calculated from the slope on a plot of velocity against the D-alanine concentration.

due to the conversion of PLP to PMP. When an amino acceptor was added to the PMP form of the enzyme, the PLP form of the enzyme was regenerated. Both half reactions were followed as to the absorption changes at 333 nm and 415 nm with a stopped-flow apparatus. However, when α-ketoglutarate was used as the substrate for the PMP form of the wild-type enzyme, the half reaction finished instantly on the addition of the substrate. The rate of conversion from PMP to PLP with α-ketoglutarate is much higher than the dead time of the apparatus used. Therefore, we used pyruvate as the amino acceptor.

The pseudo-first order rate constant (k_{app}) of the reaction depended on the concentration of the substrate, as shown by the equation,

$$k_{app} = k_{max}[S]/(K_d + [S])$$

where K_d is the dissociation constant for the enzyme-substrate complex, k_{max} is the maximal rate constant for the half reaction, and [S] is the concentration of the substrates.

The kinetic parameters thus obtained are summarized in Table II. The k_{max}/K_d values of the mutant enzymes were 2 to 3 orders of magnitude lower than those of the wild-type enzyme for both D-alanine and pyruvate. The mutations affected the K_d values to a greater degree than the k_{max} values. The k_{max} values, decreased by the Arg98 mutation, effectively recovered on the introduction of another mutation, Y88R. However, the affinity for substrates was not increased by the double mutation.

The k_{max} and K_d values of the R98M mutant enzyme for D-alanine could not be obtained, because no saturation kinetics were observed with respect to the D-alanine concentration; the k_{app} value increased proportionally with increases in the D-alanine concentration above 400 mM. However, the k_{max}/K_d value can be approximated from the slope of a plot of the k_{app} value against the D-alanine concentration: the value was only 0.033% of that of the wild-type enzyme.

DISCUSSION

D-AAT and BCAT belong to the same family of proteins, according to the classification of aminotransferases of Mehta *et al.* (*17*), but the two enzymes differ markedly from each other in substrate specificity. Stereochemical analysis of the hydrogen transfer between substrates and cofactors suggested that the substrates are bound to the active sites of the enzymes in opposite orientations (*20*). On the basis of the results of X-ray crystallographic studies, Sugio *et al.* (*15*) suggested that the side chain of an amino acid substrate is bound at a "hole" formed with the loop from Ser240 to Ser243 and the hydroxyl group of Ser180. The tertiary structure of the enzyme also suggests that Arg98 of the other subunit interacts with the α-carboxyl group of a substrate. Arg98 of D-AAT corresponds to Met107 of BCAT, as judged on linear alignment of the amino acid sequences. Therefore, we replaced Arg98 with methionine, and studied the properties of the mutant enzyme in comparison with those of the wild-type enzyme. We also constructed the Y88R/R98M mutant enzyme aiming to produce a new binding site for the α-carboxyl group of a substrate in order to compensate for the function of the lost Arg98.

Pig heart AspAT is also inhibited by high concentrations of substrates: the PMP and PLP forms are inhibited by aspartate and α-ketoglutarate, respectively (*25*). Although D-AAT was not inhibited by high concentrations of D-alanine, it was strongly inhibited by different kinds of α-keto acids: α-ketoglutarate and pyruvate. The mutation of Arg98 led to an at least one order of magnitude higher K_d value for not only pyruvate but also D-alanine (Table II). This suggests that Arg98 of the wild-type D-AAT serves as the binding site for the α-carboxyl groups of both α-keto acids and D-amino acids. If this is the case, α-keto acids and D-amino acids probably compete with each other. This competition is most probably responsible for the observed inhibition by high concentrations of α-ketoglutarate and pyruvate. The failure to observe inhibition by high concentrations of D-alanine is probably due to a difference in the affinity of the enzyme for D-alanine and α-keto acids: the K_d value for D-alanine is more than 10 times higher than that for pyruvate (Table II).

Arg88 introduced into the Y88R/R98M mutant enzyme is probably inert as a binding site for the α-carboxyl groups of substrates, because the K_d value of this double mutation enzyme for pyruvate was higher than that of the R98K mutant enzyme, and only a little lower than that of the R98M mutant enzyme (Table II). On the other hand, the k_{max} values were greatly increased by the double mutation; the function of Arg98 lost with the mutation was compensated for at least partly on the introduction of Arg88. Thus, the k_{max}/K_d values of the double mutation enzyme were much higher than those of the R98M mutant enzyme (Table II). The arginine residue introduced at position 88 probably stabilizes the transition state, which is probably the quinoid intermediate with the plane structure shown in Fig. 1, by interacting effectively with an anionic group such as the α-carboxyl group of the substrate moiety of the intermediate. Compensation for lost function by additional mutations has also been reported for ATP synthase from *E. coli* (*26*) and horseradish peroxidase (*27*).

Both D-AAT and BCAT show the same *re*-face stereospecificity for the hydrogen transfer between C-4′ of the coenzyme and C-2 of the substrate (*20*). However, D-AAT acts specifically on D-amino acids, and BCAT on L-amino

acids. Therefore, the difference in substrate specificity between D-AAT and BCAT can probably be attributed to the opposite orientations of the substrates bound at their active centers as shown in Fig. 1. Recently, Okada *et al.* clarified the tertiary structure of *E. coli* BCAT (*19*). The main chain conformation of the active site of BCAT is similar to that of D-AAT, but the active site structures of these enzymes are quite different. The putative binding site for the α-carboxyl group of a substrate of BCAT has a complex structure comprising the phenolic OH group of Tyr95 and two main-chain NH groups derived from Thr257 and Ala258. It may be interesting to design a binding site for L-amino acids to switch the stereospecificity of D-AAT on the basis of these findings.

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