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Conversion of Aspartate Aminotransferase into an L-Aspartate β-Decarboxylase by a Triple Active-site Mutation*

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The conjoint substitution of three active-site residues in aspartate aminotransferase (AspAT) of *Escherichia coli* (Y225R/R292K/R386A) increases the ratio of L-aspartate β-decarboxylase activity to transaminase activity >25 million-fold. This result was achieved by combining an arginine shift mutation (Y225R/R386A) with a conservative substitution of a substrate-binding residue (R292K). In the wild-type enzyme, Arg^{386} interacts with the α-carboxylate group of the substrate and is one of the four residues that are invariant in all aminotransferases; Tyr^{225} is in its vicinity, forming a hydrogen bond with O-3′ of the cofactor; and Arg^{292} interacts with the distal carboxylate group of the substrate. In the triple-mutant enzyme, k_{cat}' for β-decarboxylation of L-aspartate was 0.08 s^{-1}, whereas k_{cat}' for transamination was decreased to 0.01 s^{-1}. AspAT was thus converted into an L-aspartate β-decarboxylase that catalyzes transamination as a side reaction. The major pathway of β-decarboxylation directly produces L-alanine without intermediary formation of pyruvate. The various single- or double-mutant AspATs corresponding to the triple-mutant enzyme showed, with the exception of AspAT Y225R/R386A, no measurable or only very low β-decarboxylase activity. The arginine shift mutation Y225R/R386A elicits β-decarboxylase activity, whereas the R292K substitution suppresses transaminase activity. The reaction specificity of the triple-mutant enzyme is thus achieved in the same way as that of wild-type pyridoxal 5′-phosphate-dependent enzymes in general and possibly of many other enzymes, *i.e.* by accelerating the specific reaction and suppressing potential side reactions.

In the engineering of protein catalysts with new functional properties, the modification of existing enzymes provides an alternative to the production of catalytic antibodies or, in a more distant future, the *de novo* design of enzymes. Enzyme engineering may be expected to contribute to elucidating both the structural basis of the functional properties and the course of the molecular evolution. Several attempts to change the substrate specificity of an enzyme by substitution of the substrate-binding residues have succeeded (Refs. 1–9; for a review, see Ref. 6). Among the pyridoxal 5′-phosphate-dependent enzymes, aspartate aminotransferase (AspAT)[1] has been converted by multiple active-site mutations into an L-tyrosine aminotransferase (5) and by directed molecular evolution into an L-branched-chain amino acid aminotransferase (7, 8). Tyrosine phenol-lyase has been engineered by a double mutation to act as a dicarboxylic-acid β-lyase (an enzyme not found in nature) that degrades aspartate to pyruvate, ammonia, and formate (9). However, as yet, no change in the reaction specificity of an enzyme has been reported, with the exception of the conversion of papain into a peptide-nitrile hydratase (10). A change in the reaction specificity may be claimed if a new catalytic activity not inherent in the wild-type enzyme is generated and the original activity of the wild-type enzyme is suppressed to a level significantly below that of the new activity.

The pyridoxal 5′-phosphate (PLP)-dependent enzymes (B_6 enzymes) catalyze numerous reactions in the metabolism of amino acids. The B_6 enzymes are of multiple evolutionary origin and constitute a few families of homologous proteins of which the α-family is by far the largest (11). The enzyme members of the α-family, which includes AspAT, not only possess similar protein scaffolds, but most of them also share the first two steps of the reaction pathway (for a succinct introduction into PLP-dependent reaction pathways, see Ref. 12). The amino group of the incoming substrate replaces the ε-amino group of the active-site lysine residue, the internal aldimine **1** (see Scheme 1), thus being followed by the external aldimine intermediate **2,** which is then deprotonated at C-α to give the quinonoid intermediate **3**. Only in the subsequent step do the reaction pathways of the different B_6 enzymes diverge, leading to racemization, transamination, β- and γ-elimination and replacement. It seems therefore feasible to make the quinonoid intermediate **3** in a given enzyme adopt an alternative reaction course by substituting few critical active-site residues.

Aspartate aminotransferase is the most extensively studied B_6 enzyme. The homodimeric enzyme (2 × 400 amino acid residues) catalyzes the reversible transfer of the amino group of aspartate or glutamate to the cognate oxo acids. A detailed mechanism of action has been derived from combined biochemical and crystallographic data (13). In a previous study, we have generated L-aspartate β-decarboxylase activity in AspAT of *Escherichia coli* by introducing a double active-site mutation (14). AspAT Y225R/R386A β-decarboxylated L-aspartate to L-alanine with k_{cat}' = 0.08 s^{-1}, *i.e.* 1330-fold faster than the wild-type enzyme. However, transaminase activity, despite a decrease by 3 orders of magnitude, still exceeded β-decarboxylase activity by a factor of 2.5.

Here we searched for a third mutation that, if introduced

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[1] The abbreviations used are: AspAT, aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; B_6 enzyme, PLP (vitamin B_6)-dependent enzyme; PMP, pyridoxamine 5′-phosphate.

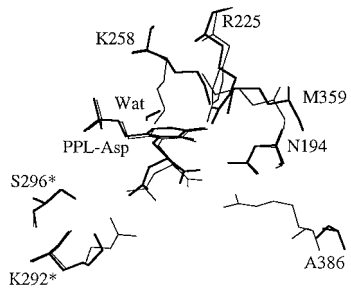


FIG. 1. **Stereo view of the active site of the 5′-phosphopyridoxyl L-aspartate complexes of AspAT Y225R/R292K/R386A (*thick lines*) and wild-type AspAT (*thin lines*).** The *asterisks* denote residues from the adjacent subunit. Gly^{38} and Ile^{37} were omitted for clarity. The orientation of the small domain significantly differed in the two structures (residues 17, 18, 360, 382, and 386; *lower right*): 5′-phosphopyridoxyl L-aspartate (*PPL-Asp*) locks wild-type AspAT in the closed conformation, whereas the triple-mutant stays in the open conformation. A water molecule (*Wat*) is found in the triple-mutant structure close to the position of a water molecule that, in mitochondrial AspAT, is assumed to carry out hydrolysis of the ketimine intermediate (V. N. Malashkevich and J. N. Jansonius, unpublished data).

into AspAT Y225R/R386A, would decrease further transaminase activity without affecting β-decarboxylase activity. The only mutation among many tested that brought about this effect was the replacement of the second active-site arginine residue, *i.e.* Arg^{292} (a residue of the adjacent subunit of the AspAT homodimer) with lysine. In the wild-type enzyme, Arg^{292} binds the distal carboxylate group of the substrate (Fig. 1). The single R292K mutation had been previously found to decrease transaminase activity to 0.2% of that of the wild-type enzyme (15). In the triple-mutant enzyme, β-decarboxylase activity indeed exceeded transaminase activity by a factor of 8.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis and Enzyme Purification—Oligonucleotide-directed mutagenesis of the wild-type *aspC* gene of *E. coli* inserted into the BS M13 vector (16) was performed with the mutagenesis kit from Bio-Rad. The mutations were confirmed by determination of the nucleotide sequences. The mutated DNAs were expressed in the AspAT-deficient *E. coli* strain TY103 (17) with the expression vector pKDHE19 (18). Wild-type and mutant enzymes were purified with previously described chromatographic procedures. Fractions containing pure AspAT were pooled, concentrated, and reconstituted with coenzyme as described (19).

Determination of Protein Concentration and Aminotransferase Activity—The concentration of the purified enzymes in the PLP form was determined spectrophotometrically at 280 nm using the molar absorption coefficient of the subunit, $\epsilon = 4.7 \times 10^4\ M^{-1}\ cm^{-1}$ (20). Kinetic parameters for aminotransferase activities of AspAT mutants and the wild-type enzyme were measured in a coupled assay with malate dehydrogenase containing 40 mM L-aspartate plus 20 mM 2-oxoglutarate as substrates for the wild-type enzyme and 100 mM L-aspartate plus 50 mM 2-oxoglutarate for the mutant enzymes. The values of k_{cat} refer to subunit concentrations. The K_m' values for L-aspartate and 2-oxoglutarate were measured at fixed concentrations of 2-oxoglutarate (50 mM) and L-aspartate (200 mM), respectively.

For measuring the consumption and production of oxo acids, the enzymes (0.45 mM, subunit concentration) were incubated with 200 mM L-aspartate and 8 mM oxalacetate in 250 mM 4-methylmorpholine (pH 7.5) at 25 °C. Samples (40 μl) were deproteinized at different time intervals with 1 M perchloric acid (final concentration) and neutralized with potassium hydroxide. Oxalacetate and pyruvate were determined separately by consumption of NADH in the presence of malate dehydrogenase and lactate dehydrogenase, respectively. The β-decarboxylation of oxalacetate in the absence of enzyme ($t_{½}$ = 60 min under the conditions detailed in the legend of Fig. 2 with 0.45 mM PLP added) was neglected in the calculations of k_{cat} for the different enzyme variants.

Measurement of Rates of β-Decarboxylation and Other Side Reactions—AspATs were incubated with amino acid and their cognate oxo acid in 250 mM 4-methylmorpholine (pH 7.5). High buffer concentrations are needed in the assays because CO_2 is released in the β-decarboxylation reaction. The β-decarboxylase activity of the two mutant enzymes is sensitive to pH; a deviation by 0.5 from the optimum at pH 7.5 decreases the activity by 50% (data not shown). After addition of 0.5 μmol of L-valine as internal standard, 20-μl deproteinized samples of the reaction mixture were derivatized with 2-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and analyzed as described previously (15, 21).

For the determination of the rates of desulfination of L-cysteine sulfinate, the enzymes (0.45 mM, subunit concentration) were incubated with 100 mM L-cysteinesulfinic acid and 50 mM 2-oxoglutarate in 200 mM 4-methylmorpholine (pH 7.5) at 25 °C, and the production of alanine was measured as described above. To check which pathway of β-elimination the enzymes were following (see Scheme 1), the same experiments were performed in the presence of 45 units/ml lactate dehydrogenase and 50 mM NADH to trap any pyruvate produced by hydrolysis of the ketimine intermediate **9** (see Scheme 1). Transamination of L-cysteine sulfinate was quantified by the increase in the concentration of L-glutamate produced by the reaction of the PMP form of the enzyme with oxoglutarate.

X-ray Crystallographic Structure Determination—Crystals of AspAT Y225R/R292K/R386A were grown with the hanging drop technique. A solution containing 15 mg/ml protein, 2 mM 5′-phosphopyridoxyl L-aspartate (13), and 50 mM 4-methylmorpholine (pH 7.5) was mixed 1:1 with reservoir solution containing 1.98 M ammonium sulfate, 2% (w/v) polyethylene glycol (M_r 400), and 200 mM 4-methylmorpholine (pH 7.5). Crystals grew to a maximum size of 0.2 × 0.4 × 1.5 mm in ~4 weeks. Nucleation and crystal growth proved more problematic than in the case of wild-type AspAT, probably indicating a less stable conformation of the protein. As found before for crystals of wild-type and mutant *E. coli* AspATs (14, 22), the crystals belong to space group $P2_1$ with unit cell dimensions a = 88.03 Å, b = 80.32 Å, c = 87.82 Å, and β = 119.85 °.

Diffraction data were collected to a resolution of 2.16 Å, using a MARresearch imaging plate mounted on a modified Elliott GX20 rotating copper anode generator. Raw data were processed with MOSFLM (23). Images were scaled with SCALA and AGROVATA and reduced to structure factors with TRUNCATE from the CCP4 Program Suite (24). A total of 170,054 measured reflections were merged together with R_{sym} = 0.082 to give rise to the data set of 50,068 independent reflections, which is 87.7% complete to 2.16-Å resolution. The structure of the 5′-phosphopyridoxyl aspartate complex of AspAT Y225R/R292K/R386A was solved by molecular replacement with the program AMORE (24), using the refined structure of the open form of the wild-type enzyme (22) as search model, and refined with TNT (25) to r = 0.19. The root mean square deviations of the bond lengths, bond angles, and planes from the ideal values were 0.011 Å, 1.35°, and 0.01 Å, respectively.

Molecular Dynamics Calculation—The simulations of the quinonoid intermediates **3** (see Scheme 1) were performed as described previously (14). The cell multipole method (26) was used instead of a cutoff for the nonbonded interactions. The modeled structure of the triple-mutant enzyme was obtained by the replacement of Arg^{292} with a lysine residue in the double-mutant crystal structure. The systems were relaxed by 4000 steps of energy minimization. The amino acid residues and water molecules beyond a distance of 11 Å from the coenzyme-substrate adduct were kept fixed during the following 100 ps of simulation at 300 K.

RESULTS

L-Aspartate β-Decarboxylase Activity—The introduction of the additional mutation R292K into AspAT Y225R/R386A did not affect its L-aspartate β-decarboxylase activity (Fig. 2); the k_{cat} and K_m' values of the triple- and double-mutant enzymes are the same (Table I). Two different pathways to produce L-alanine from L-aspartate are possible, *i.e.* direct β-decarboxylation of L-aspartate (**7** → **8** → **1** in Scheme 1) or β-decarboxylation coupled with transamination (**7** → **9** → **5**), producing pyruvate, which, by transamination, may be converted to L-alanine. To determine the partition ratio of the two pathways, the consumption of oxalacetate (consumed in the reaction with the PMP form of the enzyme **5** to produce the PLP form **1** and L-aspartate) and the production of pyruvate in the presence of L-aspartate and oxalacetate (conditions as described in the legend to Fig. 2) were followed in parallel with the β-decarboxylation of L-aspartate (Table II). Both AspAT Y225R/R386A and AspAT Y225R/R292K/R386A produced pyruvate with a k_{cat} of only 0.01 s^{-1}, corresponding to a partition ratio (**7** → **8** *versus* **7** → **9**) of 8. In the wild-type enzyme, production of

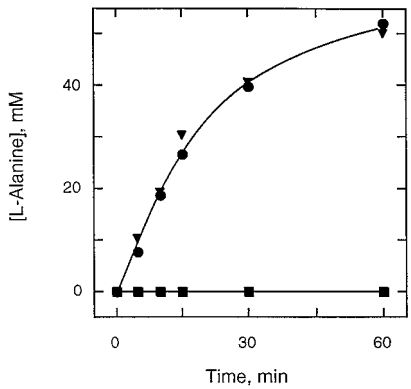


FIG. 2. **β-Decarboxylation of L-aspartate catalyzed by AspAT Y225R/R292K/R386A (●), AspAT Y225R/R386A (▼), and wild-type AspAT (■).** Enzymes (0.45 mM, subunit concentration) were incubated with 200 mM L-aspartate and 8 mM oxalacetate in 250 mM 4-methylmorpholine (pH 7.5) at 25 °C. For details of the detection of L-alanine, see "Experimental Procedures."

pyruvate by far exceeded that of L-alanine. Probably, most of the L-alanine produced by the wild-type enzyme was formed by transamination of pyruvate.

Replacement of Arg^{292} in AspAT Y225R/R386A with a glutamate or valine residue led to 16- and 100-fold decreases in k_{cat} for β-decarboxylation, respectively (Table I). Arg^{292} was substituted with glutamate to introduce a negative charge that might destabilize the β-carboxylate group (29, 30) and thus enhance β-decarboxylation. If the ratio of β-decarboxylase to transaminase activity is considered rather than the absolute k_{cat} value, AspAT Y225R/R292E/R386A is indeed a more specific L-aspartate β-decarboxylase than its counterpart with the R292K substitution, its β-decarboxylase activity being 100 times higher than its transaminase activity (Table I). Replacement of Arg^{292} with tyrosine eliminated the positive charge, whereas the phenolic hydroxy group could still form a hydrogen bond with the β-carboxylate group of the substrate and thus maintain the binding function. However, AspAT Y225R/R292Y/R386A proved to have very low affinity for the substrates and no measurable β-decarboxylase activity.

Transaminase Activity—The replacement of Arg^{292} with lysine, glutamate, valine, or tyrosine in AspAT Y225R/R386A led to a further marked decrease in k_{cat} for transamination (Table I). However, only with the R292K substitution as the third mutation was β-decarboxylase activity maintained. The K_m values for dicarboxylic substrates of the single-, double-, and triple-mutant enzymes are invariably higher than those of the wild-type enzyme, with the exception of the single Y225R mutation, which has been reported to decrease the K_m values for C_4 and C_5 dicarboxylic substrates (14, 27, 31, 32). In AspAT Y225R/R292K/R386A in particular, the K_m values for L-aspartate and 2-oxoglutarate are, as in the Y225R/R386A double-mutant enzyme, seven and four times higher, respectively, than in the wild-type enzyme.

AspAT Y225R/R292E/R386A was also tested for activity toward L-lysine, L-arginine, and L-ornithine. A very slow transamination reaction of L-lysine with an initial rate of 0.001 s^{-1} could be detected. Such an effect of a negative charge at position 292 has been reported previously (33–35). Under the same conditions, no reaction of L-lysine with the wild-type enzyme was observed. None of the mutant enzymes showed any measurable reaction other than transamination toward D/L-glutamate, D-aspartate, L-tyrosine, or L-serine and their cognate oxo acids.

Desulfination of L-Cysteine Sulfinate—Wild-type AspAT catalyzed the transamination of L-cysteine sulfinate at a very high rate. As a side reaction, elimination of sulfinate produced L-alanine (Table II). Both AspAT Y225R/R386A and AspAT Y225R/R292K/R386A showed a reaction specificity that was inverse to that of the wild-type enzyme, desulfination of L-cysteine sulfinate being by an order of magnitude faster than its transamination reaction. The double mutation increased desulfination activity 3-fold and decreased transaminase activity toward L-cysteine sulfinate by 4 orders of magnitude. The introduction of the third mutation (R292K) reduced both desulfination and the transamination activity of the double-mutant enzyme by an order of magnitude. Lactate dehydrogenase plus NADH had no effect on k_{cat} of desulfination by the wild-type and mutant AspATs, indicating that L-alanine is produced by direct desulfination of L-cysteine sulfinate rather than through formation of pyruvate followed by transamination.

Crystal Structure—The 5′-phosphopyridoxyl aspartate complex of the triple-mutant enzyme (Y225R/R292K/R386A) was found in the open conformation (Fig. 1). In the wild-type enzyme, binding of dicarboxylic substrates or inhibitors induces the closed conformation of the enzyme, in which water molecules are excluded from the vicinity of the Schiff base (13). The open conformation of the triple-mutant enzyme allows water molecules to enter the active site in the presence of the substrate analog. Lys^{258}, which is responsible in the wild-type enzyme (13) for the deprotonation at C-α and reprotonation at C-4′ of the coenzyme (Scheme 1), has moved away from its position near these atoms, where a water molecule is now found. The amino group of Lys^{258} is within hydrogen-bonding distance to Arg^{225}. Lys^{292} does not interact with the distal carboxylate group of the aspartate moiety; it forms a hydrogen bond with Ser^{296} instead, whereas a water molecule occupies its original position. The electron density of the aspartate moiety is highly disordered due to the lack of Arg^{292} and Arg^{386}, which are key residues for substrate binding in the wild-type enzyme. Nevertheless, the coenzyme-substrate adduct maintains a conformation that allows elimination of the proton at C-α, the C-α–H bond together with the imine nitrogen staying in a plane orthogonal to the plane defined by the coenzyme ring (13, 36).

Molecular Dynamics Simulations—In the simulation of the external aldimine intermediate based on the crystal structure of AspAT Y225R/R292K/R386A (Fig. 1), Lys^{258} did not displace the intervening water molecule and approach C-α. This situation most probably is due to a crystal artifact as it would correspond to a catalytically inactive enzyme. The dynamics calculations for the quinonoid intermediate of the triple-mutant enzyme were therefore based on the crystal structure of AspAT Y225R/R386A (14), in which Arg^{292} had been replaced with a lysine residue. In this case, Lys^{258} stayed close enough to C-α and C-4′ for acting as the acid-base group in the tautomerization from aldimine **2** to ketimine **4**. In the molecular dynamics simulation of the quinonoid intermediate of AspAT Y225R/R292K/R386A, as in those of AspAT Y225R/R386A, a hydrogen bond between Arg^{225} and the imine nitrogen atom is formed (Fig. 3). During the simulation, this hydrogen bond exists only 5% of the time in AspAT Y225R, whereas it is present 35% of the time in the double- and triple-mutant enzymes. In all AspATs containing the Y225R mutation, the proximity of the positively charged guanidinium group repulses the protonated Lys^{258}. Its longer distance from C-4′ of the coenzyme hinders reprotonation of that atom and might underlie the decrease in transaminase activity. In the simulated structures of the quinonoid intermediate of the wild-type enzyme, Lys^{258} remains positioned above the imine nitrogen at almost equal distance from C-α and C-4′.

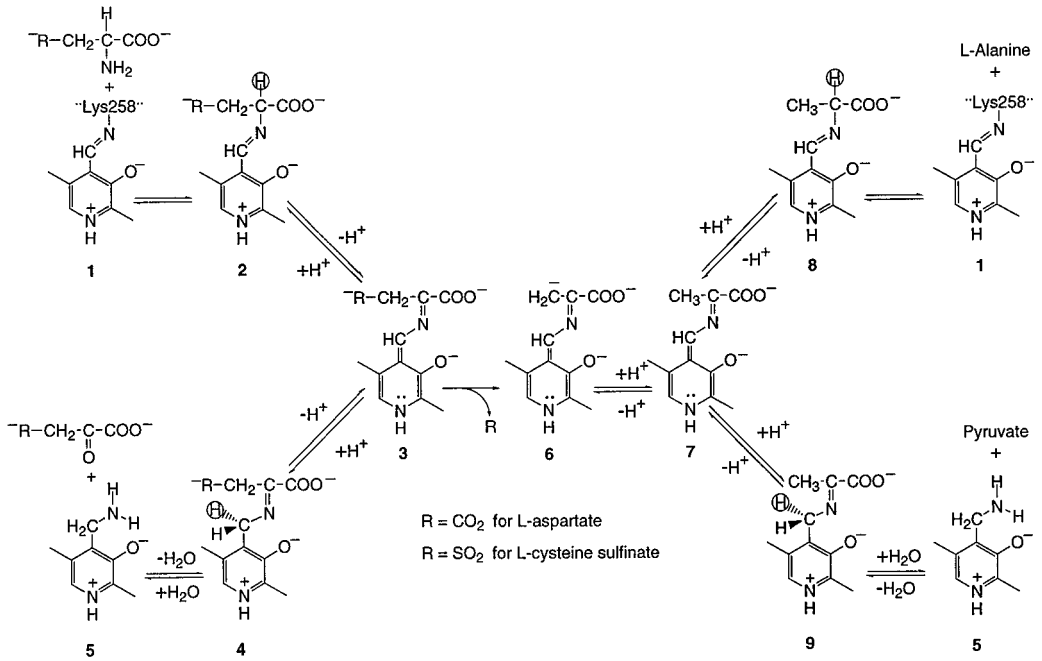
TABLE I

Kinetic parameters for β-decarboxylase and transaminase activities of wild-type and mutant AspATs

The β-decarboxylase assay was carried out in 200 mM L-aspartate plus 8 mM oxalacetate in 250 mM 4-methylmorpholine (pH 7.5). Transaminasc activity was measured in the presence of 20 mM L-aspartate plus 20 mM 2-oxoglutarate in 50 mM 4-methylmorpholine (pH 7.5) for the wild-type enzyme and 100 mM L-aspartate plus 50 mM 2-oxoglutarate in 100 mM 4-methylmorpholine (pH 7.5) for the mutant enzymes. Because the transaminase activity of AspAT Y225R/R292E/R386A was too low to be analyzed with a coupled assay, the transformation of the PLP form of the enzyme into the PMP form upon addition of 100 mM L-aspartate was followed spectrophotometrically instead. The disappearance of the PLP form and the appearance of the PMP form of the enzyme were recorded at 360 and 330 nm, respectively. All reactions were run at 25 °C. One double-mutant enzyme, AspAT Y225R/R292K, was not expressible in *E. coli* TY103. In the cell crude extract, no soluble enzyme could be detected on SDS-polyacrylamide gel after silver staining.

AspAT	β-Decarboxylation k_{cat}	Transamination k_{cat}	K_m(Asp)′	K_m(OG)′[a]
	s^{-1}	s^{-1}	mM	mM
Y225R/R292K/R386A	0.08	0.01	8.8	0.8
Y225R/R292E/R386A	0.005	5×10^{-5}	ND	ND
Y225R/R292V/R386A	8×10^{-4}	0.051	6.8	0.36
Y225R/R292Y/R386A	BD	0.025	200	14
Y225R/R386A	0.08	0.19	8.3	0.8
R292K/R386A	7.5×10^{-4}	0.094	14	0.25
Y225R	BD	0.45	0.83[b]	0.36[b]
R292K	1.8×10^{-3}	0.5[c]	14[c]	2.7[c]
R386A	BD	0.33	15	5
Wild-type	6×10^{-5} [d]	180	1.2	0.2

[a] OG, 2-oxoglutarate; ND, not determined; BD, below detection limit.
[b] From Ref. 27.
[c] From Ref. 15.
[d] Most of the L-alanine produced in the presence of the wild-type enzyme is probably due to transamination of pyruvate formed by β-decarboxylation of oxalacetate.



SCHEME 1. **Reaction pathways of enzymic transamination and β-replacement.**

DISCUSSION

The triple mutation Y225R/R292K/R386A brings about a switch in the reaction specificity of *E. coli* AspAT. The conjoint R386A and Y225R substitutions enhance the very low L-aspartate β-decarboxylase activity of the wild-type enzyme and decrease transaminase activity. Measurements of the activity of the pertinent single- and double-mutant enzymes other than Y225R/R386A confirmed that the increase in β-decarboxylase activity strictly depends on the combined effects of the R386A and Y225R substitutions (Table I). This double mutation amounts to a shift of an arginine residue from position 386 to position 225. The third mutation, replacement of Arg^{292} with lysine, selectively lowers transaminase activity to one-eighth of β-decarboxylase activity. Together, the three mutations increase the ratio of β-decarboxylase to transaminase activity >25 million-fold. Molecular dynamics simulations of the wild-type and triple-mutant enzymes based on crystal structures suggested, as previously for the double-mutant enzyme, that Arg^{225} makes, in addition to the hydrogen bond with O-3′ of the coenzyme, a second hydrogen bond with the imine nitrogen of the quinonoid intermediate. In the wild-type enzyme, the imine nitrogen is not engaged in a hydrogen bond, whereas O-3′ forms a hydrogen bond with Tyr^{225}. The single Y225R mutation is apparently not sufficient to give rise to the formation of a hydrogen bond from Arg^{225} to the imine nitrogen atom (14). The mutation has been found to decrease the pK_a of the internal aldimine from 6.8 in the wild-type enzyme to 6.2 due to a strong hydrogen bond of Arg^{225} with O-3′ (27), which pulls electrons out of the π-system extending from the pyridine ring of PLP to the aldimine bond. The imine nitrogen might thus become a too weak nucleophile to accept a hydrogen bond from Arg^{225}. The replacement of Arg^{386} by an alanine residue has

TABLE II

Comparison of k_{cat} values for β-decarboxylation and transamination with those of β-desulfination

Desulfination activity was determined by incubating 0.45 mM enzyme (subunit concentration) with 100 mM L-cysteine sulfinate and 50 mM 2-oxoglutarate in 200 mM 4-methylmorpholine (pH 7.5) at 25 °C. Transamination of L-cysteine sulfinate was measured by the amount of L-glutamate produced in the regeneration of the PLP form from the PMP form of the enzyme (for details, see "Experimental Procedures").

AspAT	k_{cat}				
	L-Aspartate			L-Cysteine sulfinate	
	β-Decarboxylation[a]	Transamination[a]	Pyruvate production	β-Desulfination	Transamination
		s^{-1}			
Y225R/R292K/R386A	0.08	0.01	0.01	0.02	0.002
Y225R/R386A	0.08	0.19	0.01	0.165	0.024
Wild-type	6×10^{-5}	180	0.0016	0.05	512[b]

[a] Values are taken from Table I.
[b] From Ref. 28.

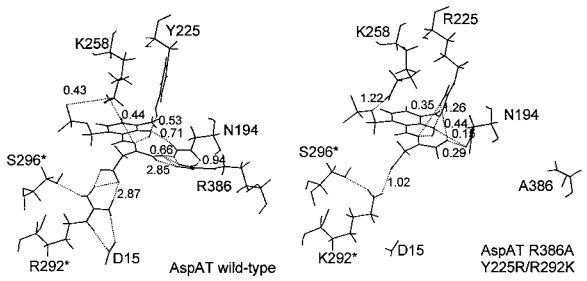


FIG. 3. **Hydrogen bonds in the active sites of the quinonoid intermediate 3 of wild-type and mutant AspATs during the molecular dynamics simulation.** The substrate is L-aspartate. The models represent the averaged structures of the last 10 ps of 100-ps simulations. The *numbers* indicate the fraction of time during which the hydrogen bond exists during the simulation. Numbers >1 are the sums for all possible hydrogen bonds of the same atom.

been reported previously to disrupt the hydrogen-bonding network $Arg^{386} \rightarrow Asn^{194} \rightarrow$ O-3′ (37) and thus to allow the electrons of O-3′ to flow into the π-system. As a consequence, the imine nitrogen in the double-mutant enzyme might engage in a hydrogen bond with Arg^{225}. A similar effect of the Y225R/R386A mutations might be operative in the quinonoid intermediate (14). The O-3′–Arg^{225} and imine nitrogen–Arg^{225} hydrogen bonds may be assumed to reinforce the electron sink capacity of the π-system of imine and the cofactor pyridine ring to such an extent that, even after deprotonation at C-α, it remains effective enough to stabilize carbanion **6** (Scheme 1), produced by β-decarboxylation. Other factors that might favor β-decarboxylation, such as the angle of the C-β–C-γ bond relative to the coenzyme-imine π-system (36) and negative electrostatic potentials around the β-carboxylate group that might promote electron delocalization (38), could not be verified; neither the bond angles nor the electrostatic potentials in AspAT Y225R/R292K/R386A were significantly different from those in the wild-type enzyme.

The decrease in transaminase activity observed in both AspAT Y225R/R386A and AspAT Y225R/R292K/R386A might be due to the repulsion between Lys^{258} and Arg^{225} impairing the reprotonation of the quinonoid intermediate **3** at C-4′. A possible explanation for the further decrease brought about by the third mutation (R292K) is provided by the recent crystallographic analysis of three reaction intermediates of the wild-type enzyme **7** that has shown a water molecule to be positioned near C-α in the ketimine intermediate **4**; this water molecule is supposed to effect the hydrolysis of the ketimine.[2] Its nucleophilicity might be enhanced by a hydrogen-bonding network comprising Tyr^{70}, Lys^{258}, and Gly^{38}. The effect of these interactions is maximal if the active site is in the closed conformation because the rotation of the small domain brings Gly^{38} into a position where it can take part in this network. The wild-type enzyme assumes the closed conformation on binding a dicarboxylic amino or oxo acid to the two active-site arginine residues, Arg^{292} and Arg^{386}. By interacting with the substrate, the two arginines are pulled toward each other, inducing the domain movement (13, 22, 39). In AspAT Y225R/R292K/R386A, the mutation of both arginine residues prevents the enzyme from adopting a closed conformation (Fig. 1), with the consequence that the water molecule might not be reactive enough to attack C-α. In AspAT Y225R/R386A, with only one substrate-binding arginine missing, the syncatalytic closure of the active-site cleft is partially retained (14).

The importance of the domain movement for the reactivity of the catalytic water molecule in the hydrolysis of the ketimine intermediate might also explain the reaction pathway of both β-decarboxylation and β-desulfination. The amino acid L-cysteine sulfinate is a dianion like aspartate and is a physiologic substrate for AspAT (40, 41). The reaction specificity of both AspAT Y225R/R386A and AspAT Y225R/R292K/R386A toward L-cysteine sulfinate is inverse to that of the wild-type enzyme. The mutant enzymes desulfinate this substrate faster than they undergo the transamination reaction with it. Nevertheless, similar to the wild-type enzyme and in analogy to the β-decarboxylation reaction with aspartate, they preferentially reprotonate carbanion **7** (Scheme 1) at C-α rather than C-4′ and produce L-alanine (**7** → **8** → **1**) rather than pyruvate (**7** → **9** → **5**). Conceivably, upon loss of the negatively charged β-substituent, the active site assumes the open conformation. Thus, the frequency of ketimine hydrolysis is decreased, and the partition ratio is shifted in favor of reprotonation at C-α, resulting in the production of L-alanine.

The starting point of this work was a study of the molecular evolution of B_6 enzymes (11). Within a given family, in particular in the large α-family, a clear temporal sequence of different phases in the functional specialization is evident. The common ancestor enzyme, apparently an unspecific all-rounder catalyst, first diverged into reaction-specific protoenzymes, which then diverged further and acquired substrate specificity. The last phase for most B_6 enzymes was the neutral evolution concomitant with speciation.[3] The conjoint substitution of three active-site residues that converted AspAT into an L-aspartate β-decarboxylase seems to simulate the processes that, in the first phase of molecular evolution, might have led to reaction-specific B_6 enzymes by accelerating the specific reaction and suppressing potential side reactions. To the best of our knowledge, such a clear change in reaction specificity with a remarkably high new activity (k_{cat} = 0.08 s^{-1}) has as yet only been reported for papain that was converted into a peptide-

[2] V. N. Malashkevich and J. N. Jansonius, unpublished data.

[3] Mehta, P. K., and Christen, P. (2000) *Adv. Enzymol. Relat. Areas Mol. Biol.*, in press.

nitrile hydratase by a single amino acid substitution at the active site (10).

Two features of the procedure used in this study for changing the reaction specificity might be generally applicable to B_6 enzymes and perhaps certain other enzymes as well. 1) The double mutation Y225R/R386A shifts an arginine residue from its wild-type position to another position in its immediate vicinity. Such charge-shifting double mutations may be expected not to disturb greatly the topochemistry of the active site, but to alter the electron repartition at the reaction center. 2) Arginine residues are the preferred binding groups for anionic substrates in enzymes (15). Their conservative substitution by lysine, which is slightly shorter and engages in fewer hydrogen bonds, may be expected to change the mode of binding of the substrate.

REFERENCES

1. Wilks, H. M., Hart, K. W., Freeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R., and Holbrook, J. J. (1988) *Science* **242,** 1541–1544
2. Bradley, M., Bücheler, U. S., and Walsh, C. T. (1991) *Biochemistry* **30,** 6124–6127
3. Scutton, N. S., Berry, A., and Perham, R. N. (1990) *Nature* **343,** 38–43
4. Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992) *Science* **255,** 1249–1253
5. Onuffer, J. J., and Kirsch, J. F. (1995) *Protein Sci.* **4,** 1750–1757
6. Shao, Z., and Arnold, F. H. (1996) *Curr. Opin. Struct. Biol.* **6,** 513–518
7. Yano, T., Oue, S., and Kagamiyama, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5511–5515
8. Oue, S., Okamoto, A., Yano, T., and Kagamiyama, H. (1999) *J. Biol. Chem.* **274,** 2344–2349
9. Mouratou, B., Kasper, P., Gehring, H., and Christen, P. (1999) *J. Biol. Chem.* **274,** 1320–1325
10. Dufour, E., Storer, A. C., Ménard, R. (1995) *Biochemistry* **34,** 16382–16388
11. Alexander, F. W., Sandmeier, E., Mehta, P. K., and Christen, P. (1994) *Eur. J. Biochem.* **219,** 953–960
12. Metzler, D. E. (1977) *Biochemistry*, pp. 444–461, Academic Press, Orlando, FL
13. Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., and Christen, P. (1984) *J. Mol. Biol.* **174,** 497–525
14. Graber, R., Kasper, P., Malashkevich, V. N., Sandmeier, E., Berger, P., Gehring, H., Jansonius, J. N., and Christen, P. (1995) *Eur. J. Biochem.* **232,** 686–690
15. Vacca, R. A., Giannattasio, S., Graber, R., Sandmeier, E., Marra, E., and Christen, P. (1997) *J. Biol. Chem.* **272,** 21932–21937, and references cited therein
16. Malcolm, B. A., and Kirsch, J. F. (1985) *Biochem. Biophys. Res. Commun.* **132,** 915–921
17. Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., Hiromi, K., and Kagamiyama, H. (1991) *J. Biol. Chem.* **266,** 6079–6085
18. Kamitori, S., Hirotsu, K., Higuchi, T., Kondo, K., Inoue, K., Kuramitsu, S., Kagamiyama, H., Higuchi, Y., Yasuoka, N., Kusunoki, M., and Matsuura, Y. (1987) *J. Biochem.* (*Tokyo*) **101,** 813–816
19. Vacca, R. A., Christen, P., Malashkevich, V. N., Jansonius, J. N., and Sandmeier, E. (1995) *Eur. J. Biochem.* **227,** 481–487
20. Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y., and Kagamiyama, H. (1990) *Biochemistry* **29,** 5469–5476
21. Kochhar, S., and Christen, P. (1988) *Eur. J. Biochem.* **175,** 433–438
22. Jäger, J., Moser, M., Sauder, U., and Jansonius, J. N. (1994) *J. Mol. Biol.* **239,** 285–305
23. Leslie, A. G. V. (1994) *MOSFLM Users Guide*, Medical Research Council-Laboratory of Molecular Biology, Cambridge
24. CCP4 Program Suite: Collaborative Computing Project No. 4 (1994) *Acta Crystallogr. Sect. D* **50,** 760–763
25. Tronrud, D. E., Ten Eyck, L. F., and Matthews, B. W. (1987) *Acta Crystallogr. Sect. A* **43,** 489–501
26. Greengard, L., and Rokhlin, V. I. (1987) *J. Comp. Physiol.* **73,** 325–330
27. Inoue, K., Kuramitsu, S., Okamoto, A., Hirotsu, K., Higuchi, T., Morino, Y., and Kagamiyama, H. (1991) *J. Biochem.* (*Tokyo*) **109,** 570–576
28. Furumo, N. C., and Kirsch J. F. (1995) *Arch. Biochem. Biophys.* **319,** 49–54
29. Leussing, D. L., and Raghavan, N. V. (1980) *J. Am. Chem. Soc.* **102,** 5635–5643
30. Hurley, J. H., and Remington, S. J. (1992) *J. Am. Chem. Soc.* **114,** 4769–4773
31. Goldberg, J. M., Swanson, R. V., Goodman, H. S., and Kirsch, J. F. (1991) *Biochemistry* **30,** 305–312
32. Goldberg, J. M., and Kirsch, J. F. (1996) *Biochemistry* **35,** 5280–5291
33. Cronin, C. N., and Kirsch, J. F. (1988) *Biochemistry* **27,** 4572–4579
34. Hwang, J. K., and Warshel, A. (1988) *Nature* **334,** 270–272
35. Almo, S. C., Smith, D. L., Danishefsky, A. T., and Ringe, D. (1994) *Protein Eng.* **7,** 405–412
36. Dunathan, H. C. (1966) *Proc. Natl. Acad. Sci. U. S. A.* **55,** 712–716
37. Yano, T., Mizuni, T., and Kagamiyama, H. (1993) *Biochemistry* **32,** 1810–1815
38. Gallagher, T., Snell, E. E., and Hackert, M. L. (1989) *J. Biol. Chem.* **264,** 12737–12743
39. Picot, D., Sandmeier, E., Thaller, C., Vincent, M. G., Christen, P., and Jansonius, J. N. (1991) *Eur. J. Biochem.* **196,** 329–341
40. Parsons, B., and Rainbow, T. C. (1984) *Brain Res.* **294,** 193–197
41. Weinstein, C. L. Haschemeyer, R. H., and Griffith, O. W. (1988) *J. Biol. Chem.* **263,** 16568–16579