Conversion of Aspartate Aminotransferase into an L-Aspartate \( \beta \)-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 \( \beta \)-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, Arg386 interacts with the \( \alpha \)-carboxylate group of the substrate and is one of the four residues that are invariant in all aminotransferases; Tyr232 is in its vicinity, forming a hydrogen bond with O-3 of the cofactor; and Arg292 interacts with the distal carboxylate group of the substrate. In the triple-mutant enzyme, \( k_{\text{cat}}' \) for \( \beta \)-decarboxylation of l-aspartate was 0.08 s\(^{-1}\), whereas \( k_{\text{cat}}' \) for transamination was decreased to 0.01 s\(^{-1}\). AspAT was thus converted into an \( \alpha \)-aspartate \( \beta \)-decarboxylase that catalyzes transamination as a side reaction. The major pathway of \( \beta \)-decarboxylation directly produces l-alanine without intermed- 
iary 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 \( \beta \)-decarboxylase activity. The arginine shift mutation Y225R/R386A elicits \( \beta \)-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 \( \alpha \)-branched-chain amino acid aminotransferase (7, 8). Tyrosine phenol-lyase has been engineered by a double mutation to act as a dicarboxylic-acid \( \beta \)-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-nitrite 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 \( \alpha \)-family is by far the largest (11). The enzyme members of the \( \alpha \)-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 \( \epsilon \)-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-O 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, \( \beta \)- and \( \gamma \)-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 \times 400 amino acid residues) catalyzes the reversible transfer of the amino group of aspartate or glutamate to the cognate o xo acids. A detailed mechanism of action has been derived from combined biochemical and crystallographic data (13). In a previous study, we have generated \( \alpha \)-aspartate \( \beta \)-decarboxylase activity in AspAT of Escherichia coli by introducing a double active-site mutation (14). AspAT Y225R/R386A \( \beta \)-decarboxylated \( \alpha \)-aspartate to L-alanine with \( k_{\text{cat}}' = 0.08 \text{s}^{-1} \), i.e., 1330-fold faster than the wild-type enzyme. However, transaminase activity, despite a decrease by 3 orders of magnitude, still exceeded \( \beta \)-decarboxylase activity by a factor of 2.5.

Here we searched for a third mutation that, if introduced

\(^{1}\)The abbreviations used are: AspAT, aspartate aminotransferase; PLP, pyridoxal 5'-phosphate; B\(_6\) enzyme, PLP (vitamin B\(_6\)) dependent enzyme; PMP, pyridoxamine 5'-phosphate.
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. Arg292 (a residue of the adjacent subunit of the AspAT homodimer) with lysine. In the wild-type enzyme, Arg292 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 mutant 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—purified AspAT were pooled, concentrated, and reconstituted with coenzyme.

Determination of Protein Concentration and Aminotransferase Activity—The concentration of the purified enzymes in the PPL form was determined spectrophotometrically at 280 nm using the molar absorption coefficient of the subunit, ε = 4.7 × 10^5 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-asperate plus 20 mM 2-oxoglutarate as substrates for the wild-type enzyme and 100 mM l-asperate plus 50 mM 2-oxoglutarate for the mutant enzymes. The values of k_cat refer to subunit concentrations. The K_m values for l-asperate and 2-oxoglutarate were measured at fixed concentrations of 2-oxoglutarate (50 mM) and l-asperate (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-asperate and 8 mM oxaloacetate 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 oxaloacetate in the absence of enzyme (t_e = 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-cysteinesulfonic acid and 50 mM 2-oxoglutarate in 200 mM 4-methylmorpholine (pH 7.5) at 25 °C, and the production of alamine 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 of 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.

RESULTS

L-Aspartate β-Decarboxylase Activity—The introduction of the additional mutation R292K into AspAT Y225R/R386A did not affect its l-asperate 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-asperate are possible, i.e. direct β-decarboxylation of l-asperate (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 PPL form 1 and l-asperate) and the production of pyruvate in the presence of l-asperate and oxalacetate (conditions as described in the legend to Fig. 2) were followed in parallel with the β-decarboxylation of l-asperate (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
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 Arg292 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). Arg292 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-β-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 Arg292 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 Arg292 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 C4 and C6 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 L- 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 (15). The open conformation of the triple-mutant enzyme allows water molecules to enter the active site in the presence of the substrate analog. Lys258, which is responsible in the wild-type enzyme (13) for the deprotonation at C-α and reprotocation at C-4' of the coenzyme (Scheme I), has moved away from its position near these atoms, where a water molecule is now found. The amino group of Lys258 is within hydrogen-bonding distance to Arg292. Lys258 does not interact with the distal carboxylate group of the aspartate moiety; it forms a hydrogen bond with Ser396 instead, whereas a water molecule occupies its original position. The electron density of the aspartate moiety is highly disordered due to the lack of Arg292 and Arg398, 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), Lys258 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 Arg292 had been replaced with a lysine residue. In this case, Lys258 stayed close enough to C-α and C-4' for acting as the acid-base group in the tau-tomerization 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 Arg225 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 Lys258. Its longer distance from C-4' of the coenzyme hinders reprotocation of that atom and might underlie the decrease in transaminase activity. In the simulated structures of the quinonoid intermediate of the wild-type enzyme, Lys258 remains positioned above the imine nitrogen at almost equal distance from C-α and C-4'.
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 \(\beta\)-decarboxylase activity of the wild-type enzyme and decrease transaminase activity. 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          | \(\beta\)-Decarboxylation | Transamination | \(K_m\) (Asp) | \(K_m\) (OG) |
|----------------|---------------------------|----------------|---------------|--------------|
| Y225R/R292K/R386A | 0.08 \(s^{-1}\)            | 0.01 \(s^{-1}\)   | 8.8 \(mM\)    | 0.8 \(mM\)   |
| Y225R/R292E/R386A | 0.005 \(5 \times 10^{-5}\) | ND             | ND            | ND           |
| Y225R/R292V/R386A | 8 \(\times 10^{-4}\)       | 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 \(\times 10^{-4}\)    | 0.094          | 14            | 0.25         |
| Y225R           | 0.45                      | 0.83 \(b\)     | 0.36 \(c\)    | 2.7 \(c\)    |
| R292K           | 1.8 \(\times 10^{-3}\)    | 0.5 \(c\)      | 14 \(c\)      | 2.7 \(c\)    |
| R386A           | BD                        | 0.33           | 15            | 5            |
| Wild-type       | 6 \(\times 10^{-3}\) \(d\) | 180            | 1.2           | 0.2          |

* OG, 2-oxoglutarate; ND, not determined; BD, below detection limit.

* From Ref. 27.

* From Ref. 15.

* Most of the L-alanine produced in the presence of the wild-type enzyme is probably due to transamination of pyruvate formed by \(\beta\)-decarboxylation of oxalacetate.

**Scheme 1.** Reaction pathways of enzymic transamination and \(\beta\)-replacement.
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 FMP form of the enzyme (for details, see "Experimental Procedures").

| AspAT                          | $k_{cat}$ | l-Aspartate (±σ) | Pyruvate production | L-Cysteine sulfinate (±σ) |
|-------------------------------|----------|------------------|---------------------|---------------------------|
| Y225R/R292K/R386A             | 0.08     | 0.01             | 0.01                | 0.02                      | 0.002                        |
| Y225R/R386A                   | 0.08     | 0.19             | 0.0016              | 0.165                     | 0.024                        |
| Wild-type                     | $6 \times 10^{-5}$ | 180             |                     |                           |                             |

Values are taken from Table I.

From Ref. 28.

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**Enzyme Engineering: Change in Reaction Specificity**

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The wild-type enzyme assumes the closed conformation on binding a dicarboxylic amino or oxo acid to the two active-site arginine residues, Arg$^{225}$ 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 reprotoxinate 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 reprotoxination 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. 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.

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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 B6 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.

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