Tyrosine phenol-lyase (TPL), which catalyzes the \(\beta\)-elimination reaction of \(\L-Tyrosine\), and aspartate aminotransferase (AspAT), which catalyzes the reversible transamination reaction of the dicarboxylic amino acids to oxo acids, both belong to the \(\alpha\)-family of vitamin B\(_6\)-dependent enzymes. To switch the substrate specificity of TPL from \(\L-Tyrosine\) to dicarboxylic amino acids, two amino acid residues of AspAT, thought to be important for the recognition of dicarboxylic substrates, were grafted into the active site of TPL. Homology modeling and molecular dynamics identified Val-283 in TPL to match Arg-292 in AspAT, which binds the distal carboxylate group of substrates and is conserved among all known AspATs. Arg-100 in TPL was found to correspond to Thr-109 in AspAT, which interacts with the phosphate group of the coenzyme. The double mutation R100T/V283R of TPL increased the \(\beta\)-elimination activity toward dicarboxylic amino acids at least \(10^4\)-fold. Dicarboxylic amino acids (\(\L\)-aspartate, \(\L\)-glutamate, and \(\L\)-\(\beta\)-amino adipate) were degraded to pyruvate, ammonia, and the respective monocarboxylic acids, \(\eg\) formate in the case of \(\L\)-aspartate. The activity toward \(\L\)-aspartate \((k_{cat} = 0.21 \text{ s}^{-1})\) was two times higher than that toward \(\L\)-tyrosine. \(\beta\)-Elimination and transamination as a minor side reaction \((k_{cat} = 0.001 \text{ s}^{-1})\) were the only reactions observed. Thus, TPL R100T/V283R accepts dicarboxylic amino acids as substrates without significant change in its reaction specificity. Dicarboxylic amino acid \(\beta\)-lyase is an enzyme not found in nature.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis—**Plasmid pTZTPL (22) containing the entire coding sequence of \(C.\ freundii\) tyrosine phenol-lyase was used as template for \textit{in vitro} mutagenesis. The \(tpl\) gene was amplified by the polymerase chain reaction using the following two synthetic oligonucleotides as primers: \(5’-\text{CGCCGCTCGACATAATTATTTATACCTGGATACATGTTGATAACAGCGG-3’}\) and \(5’-\text{GCCGAGATTCACTCAGTGCTG-3’}\). The first oligonucleotide hybridizes to the 5’ part of the \(tpl\) gene and contains six histidine codons (\textit{italics}), in frame, just before the stop codon and a new \textit{SalI} site (underlined). The second oligonucleotide hybridizes to the unique \(BglII\) site (underlined) in the \(tpl\) gene upstream to the transcriptional start point. The resulting 1.9-kilobase pair polymerase chain reaction product was cut with \(BglII\) and \textit{SalI} and subcloned into the \textit{BamHI-SalI} sites of the expression vector pTZ18U (Bio-Rad) to generate pTZTPL-His. The mutants were prepared by polymerase chain reaction from pTZTPL-His using the QuikChange™ Site-directed Mutagenesis Kit from Agilent Technologies.
Stratagene and the following primer pairs: R100Ta, 5'-CCGAGAGCGATCGGCAGGCAACAACCCCG-3'; R100Tb, 5'-CGGTCTTCTCAGCCGATCGGCAGGCAACAACCCCG-3'; V283Ra, 5'-CTCGCTACACACCGGACTAAG-3'; and V283Rb, 5'-GGATCCGATCGGCAGGCAACAACCCCG-3'. The insertion of the histidine codons and the mutations was verified by cycle sequencing (Sequenase Long-Read Cycle Sequencing Kit, Epicentre Technologies) with fluorescent primers using a DNA sequencer (LICOR).

Expression and Purification—E. coli SVS270 cells were used as host for the pTZTPL- His and the mutant plasmids. The cells, grown as described previously (22), were thawed and suspended in 5 ml of Buffer A (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 0.1 mM PLP, pH 8.0) per gram of wet weight. The cells were disrupted by three passages through a French press. Cell debris was removed by centrifugation at 25,000 × g at 4 °C for 30 min. The supernatant was passed through a 0.22-μm filter and directly applied onto a 13 × 1-cm column containing 2–3 ml of nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with Buffer A. The column was washed with Buffer A containing 20 mM imidazole until A280 of the flow-through solution was below 0.01. The TPL protein was then eluted with a 30-ml gradient from 20 to 250 mM imidazole in Buffer A. The pooled TPL fractions were dialyzed extensively against 0.1 M potassium phosphate, pH 7.0, containing 0.1 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 0.1 mM PLP, purified wide-type and mutant TPLs were stable at least for 1 year when stored at −70 °C in the same buffer at a concentration of 2–5 mg/ml. All preparations were pure as indicated by SDS-polyacrylamide gel electrophoresis (10–15% PHAST-gel from Amersham Pharmacia Biotech).

Protein Determination—The concentration of purified TPLs was determined photometrically (ε280 = 8.37; Ref. 5) assuming a subunit molecular mass of 52.3 kDa (13) which takes into account the molecular mass of the His6 tag (0.84 kDa). The PLP content of the enzymes was determined from the absorption spectrum of the enzyme in 0.1 M NaOH, assuming ε350 = 6600 M−1 cm−1 (23).

Absorption Spectra of Tyrosine Phenol-lyase—Prior to recording absorption spectra, the stock enzyme was incubated with 0.5 mM PLP for 1 h at 30 °C and then separated from excess PLP on a short desalting column (NAP™5, Amersham Pharmacia Biotech) equilibrated with 50 mM potassium phosphate, pH 8.0. Absorption spectra were measured with a 8453 UV-visible diode-array spectrophotometer from Hewlett-Packard.

Measurement of β-Elimination Activity—The activity of the TPLs toward various amino acid substrates was measured using the coupled assay with lactate dehydrogenase and NADH previously described for tryptophan indole-lyase (24). The standard assay mixture contained 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 50 μM PLP, 0.2 mM NADH, 24 units of lactate dehydrogenase from bovine heart (Sigma), and varying concentrations of amino acid substrate in a final volume of 1 ml at 25 °C. The reaction was initiated by the addition of TPL and followed by the decrease in absorbance at 340 nm. Steady-state kinetic values of Vmax and Km were obtained by fitting the data to the Michaelis-Menten equation using ORIGIN software (Micoracal Software).

Measurement of Transaminase and Racemase Activity—Mutant TPLs and wild-type enzyme were incubated in 50 mM potassium phosphate, pH 8.0, with different amino acids as substrates. Samples were withdrawn at different times and immediately deproteinized with 1 M perchloric acid (25). After derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (26), the reaction samples were loaded onto a reverse-phase high pressure liquid chromatography column (Aquapore RP-300; 250 × 4.6-mm). Both pyridoxamine 5'-phosphate (PMP), produced by the single turnover half-reaction of transamination, and the products of racemization can be separated and detected photometrically at 340 nm by this sensitive assay (27). Alternatively, the increase in absorbance at 325 nm was used to follow the production of PMP.

Thin Layer Chromatography—Protein was eliminated from the reaction mixture prior to chromatography by precipitation with 1 M perchloric acid (25). Thin layer chromatography was performed on precoated silica gel plates SIL G-25 from Macherey-Nagel in n-pentyl formate/chloroform/formic acid (50:70:10, v/v). A slightly alkaline solution of bromocresol green (0.02% in ethanol) was used to develop the chromatogram. The acids appeared as yellow spots on a blue background (28).

Formate Dehydrogenase Assay—Formic acid was determined with the formate dehydrogenase assay. A kit from Boehringer Mannheim was used according to the supplier's protocol. Briefly, mutant TPLs were incubated at 25 °C in 50 mM potassium phosphate, pH 8.0, with L-aspartate as substrate. Samples were withdrawn at different times and immediately deproteinized with perchloric acid. Formate was quantitated by the increase in absorbance at 340 nm due to NAD+ reduction.

Molecular Modeling and Dynamics Simulations—The crystal structure of the holoenzyme complex with the substrate analog 3-(4-hydroxyphenyl) propionic acid (Brookhaven Protein Data Bank, code 2TPL) was used as parent structure. The substrate analog was replaced by l-tyrosine, and the external aldime form 1 (Scheme 1) was created by introducing a double bond between C4' and the nitrogen atom of the substrate. Removal of the Ca-hydrogen, change of the hybridization of the Ca atom from sp3 to sp2, and subsequent minimization led to the quinonoid intermediate 2. Molecular dynamics simulations of this intermediate were performed using the Discover program (Molecular Simulations) with the consistent valence force field. The cell multipole method was used instead of a cut-off for the nonbonded interactions. The temperature was set to 400 K. All hydrogen atoms and explicit water molecules were included in the simulations with time steps of 1 fs. At the beginning, the whole system was minimized for 2000 steps.
correspond to the quinonoid intermediates with L-tyrosine and L-aspartate, respectively, as obtained by molecular modeling and dynamics simulations (for details, see "Experimental Procedures"). The Co atoms of Lys-257, Arg-217, Arg-404, and Val-283 of TPL were superimposed with the Co atoms of the corresponding Lys-258, Tyr-225, Arg-386, and Arg-282, respectively, in AspAT. The amino acid residues in TPL corresponding to Arg-292 and Thr-109 in AspAT are marked with an asterisk.

The outer shell was then kept fixed, and another 2000 steps of minimization were applied. This was followed by a molecular dynamics simulation, which was initialized at 400 K for 1000 fs. After this initialization, the outer shell was again kept fixed. The simulation was continued for a total time of 20 ps. Every 100 fs the potential energy was analyzed. Within each picosecond, only the structure with the lowest potential energy was stored, resulting in a total of 20 low energy structures. All these 20 structures were then minimized for 2500 steps. The resulting minimized structures were found to be generally quite similar, and one of these corresponding to the average structure was chosen as starting point for all further simulations. The modeled structure of the wild-type enzyme with L-aspartate as substrate was obtained by replacement of the leaving group of the substrate relative to the planar coenzyme-substrate adduct did not appear to be optimum for a hydrophobic active site of the enzyme (Table 1). The His-tagged TPL R100T/V283R enzyme and the single mutant TPL R100T were purified and used for analysis. The single mutant V283R enzyme, however, could not be expressed as soluble protein. The PLP content of wild-type TPL and mutant TPLs—The C-terminal His₄ tag did not interfere with the β-elimination activity of the enzyme (Table 1). The His-tagged TPL R100T/V283R enzyme and the single mutant TPL R100T were purified and used for analysis. The single mutant V283R enzyme, however, could not be expressed as soluble protein. The PLP content of

**FIG. 1.** Superposition of the active-site structures of *C. freundii* TPL (thick lines) and *E. coli* AspAT (thin lines). The structures correspond to the quinonoid intermediates with L-tyrosine and L-aspartate, respectively, as obtained by molecular modeling and dynamics simulations (for details, see "Experimental Procedures"). The Co atoms of Lys-257, Arg-217, Arg-404, and Val-283 of TPL were superimposed with the Co atoms of the corresponding Lys-258, Tyr-225, Arg-386, and Arg-282, respectively, in AspAT. The amino acid residues in TPL corresponding to Arg-292 and Thr-109 in AspAT are marked with an asterisk.

**RESULTS**

**Design Strategy**—In order to change the substrate specificity of TPL in favor of dicarboxylic amino acids, we compared TPL with AspAT using homology modeling and molecular dynamic simulations. The specificity of AspAT for dicarboxylic amino acids and oxo acids seems to be based primarily on the salt bridge-hydrogen bond interaction of the side chain of Arg-292 (of the adjacent subunit) with the distal carboxylate group of these substrates (12). In agreement with this notion, Arg-292 is conserved among all AspATs (29). Since the sequence identity between AspAT and TPL is too low (23%) to allow the use of standard alignment algorithms, comparison of their three-dimensional structures (13, 30) by superposition (Fig. 1) and with the program DALI (Fig. 2) was used to identify in TPL the residue corresponding to Arg-292 in AspAT. Val-283 in TPL seems to occupy the same position as Arg-292 in AspAT. Another significant difference in the active sites of these two enzymes is the replacement of a residue interacting with the phosphate group of the coenzyme. Arg-100 in TPL apparently corresponds to Thr-109 in AspAT which is also conserved among all AspATs (Figs. 1 and 2; Ref. 29).

Molecular modeling showed that the quinonoid adduct of L-aspartate and PLP can be sterically accommodated in the active site of wild-type TPL (Fig. 3). However, the orientation of the leaving group of the substrate relative to the planar coenzyme-substrate adduct did not appear to be optimum for a β-elimination reaction. Positively charged Arg-100 in the hydrophobic active site of TPL interacted with the distal carboxylate group of dicarboxylic substrates and thus perturbed the required orthogonal orientation of the plane defined by Coα, Cβ, and Cγ of the amino acid substrate relative to the plane defined by the π system of the coenzyme-substrate adduct including Cβ (Scheme 1; Ref. 33). This notion agrees with previous studies by Faleev et al. (34) who have reported that aspartic and glutamic acid are not substrates but, in view of the low hydrophobicity of their side chains, anomalously strong inhibitors of TPL ($K_i = 3.5$ and $5.0 \text{ mM}$, respectively). We concluded that the introduction of an arginine residue into position 283 of TPL together with the substitution of Arg-100 with an uncharged residue, i.e. the double mutation R100T/V283R, might mimic the binding site for dicarboxylic substrates of AspAT and thus result in a corresponding alteration in the substrate specificity of TPL.

**Expression of Wild-type TPL and Mutant TPLs**—The C-terminal His₄ tag did not interfere with the β-elimination activity of the enzyme (Table 1). The His-tagged TPL R100T/V283R enzyme and the single mutant TPL R100T were purified and used for analysis. The single mutant V283R enzyme, however, could not be expressed as soluble protein. The PLP content of
the mutant proteins was found to be 1 mol/mol of subunit, as has been shown previously for wild-type TPL (36). The UV-visible spectrum of the PLP form of the mutant enzymes is almost identical to that of the wild-type enzyme. Apparently, the topochemistry of the PLP-binding site is not significantly altered by the mutations.

Wild-type TPL in the presence of L-tyrosine exhibits a visible absorbance peak at about 500 nm attributable to the quinonoid adduct. From Ref. 43.

### Changes in Substrate Specificity—TPL R100T/V283R and TPL R100T were tested for β-elimination activity toward L-tyrosine and dicarboxylic amino acids of various lengths (Table I). The $k_{cat}$ value of TPL R100T/V283R toward L-tyrosine was decreased 30-fold as compared with wild-type TPL without significant change in the $K_m$ value. When TPL R100T/V283R was tested for activity toward dicarboxylic amino acids using the coupled assay with lactate dehydrogenase and NADH, pyruvate was detected in the reaction mixtures. Thin layer chromatographic analyses confirmed the production of pyruvate. A yellow spot, the $R_f$ value of which was the same as that of authentic pyruvate, was detected on the plate as the unique oxo acid product of the enzymic reactions with all dicarboxylic substrates. No 2-oxobutyric acid, which possibly might have been produced by a γ-elimination reaction of L-glutamate, was detected.

The expected products of the β-elimination reaction of the dicarboxylic substrates L-aspartate, L-glutamate, and L-2-aminoadipate are pyruvate, ammonia, and the monocarboxylic acids formate, acetate, and propionate, respectively. In the case of L-aspartate, formate was identified and determined using the coupled assay with formate dehydrogenase and NAD$^+$.

### Table I

| Substrate | Wild-type enzyme | R100T | R100T/V283R |
|-----------|------------------|-------|-------------|
|           | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
| L-Tyrosine | 3.7 | 0.2 | $1.85 \times 10^4$ | 0.54$^b$ | 8.3$^b$ | 65.1$^b$ |
| L-Aspartate | Activity below detection | 0.035 | 56 | 0.63 | 0.21 | 54 |
| L-Glutamate | Activity below detection | 0.08 | 15 | 5.4 | 0.10 | 5.3 |
| L-2-Aminoadipate | Activity below detection | 0.095$^b$ | - | - | 0.19$^b$ | - |
| 3-Chloro-L-alanine | 3.0$^c$ | 1.7$^c$ | $1.8 \times 10^3$ | 1.13 | 19 | 59 |

$^a$ The concentration ranges of the tested amino acids were as follows: 10–200 mM for dicarboxylic amino acids; 0.2–2 mM for L-tyrosine.

$^b$ Values were also higher (up to 3 times, in the case of L-glutamate) as compared with the double mutant enzyme.

$^c$ Saturation was not apparent within the concentration range tested. Values measured at a substrate concentration of 120 mM.

$^d$ From Ref. 43.

### Changes in Reaction Specificity—To determine the loss of activity by TPL R100T/V283R, wild-type TPL in the presence of L-tyrosine exhibits a visible absorbance peak at about 500 nm attributable to the quinonoid adduct.

**Fig. 3.** Superposition of the active-site structure of TPL with L-tyrosine (thick lines) and the modeled structure with L-aspartate as substrate (thin lines). The quinonoid intermediates are shown (for details, see "Experimental Procedures").
The reaction mixture contained 1 μM enzyme, 100 mM l-aspartate and 100 μM PLP in 50 mM potassium phosphate (pH 8.0). At the indicated times, samples were withdrawn from the incubation mixture and analyzed, as described under "Experimental Procedures."

| Time of reaction | Products | Products | Formate |
|------------------|----------|----------|---------|
| min              |          |          |         |
| 5                | 44.1 μM  | 43.8 μM  |         |
| 10               | 88.2 μM  | 87.7 μM  |         |

*Pyruvate was measured using the coupled assay with lactate dehydrogenase and NADH.

**Discussion**

X-ray crystallographic analysis has identified Arg-292 as the key residue in determining the specificity of AspAT for dicarboxylic substrates (12) through direct salt bridge-hydrogen bond interactions with the β- or γ-carboxylate groups of the substrate. Indeed, replacement of Arg-292 with an aspartate residue has converted E. coli AspAT from an anionic to a very slow cationic amino acid transaminase (37). Similarly, substitution of Arg-292 with valine or leucine has been found to switch the specificity in favor of aromatic amino acids (38).

Recently, the importance of the guanidinium group of arginine has also been pointed out by the marked decrease in both the affinity and the catalytic activity toward dicarboxylic substrates upon replacement of Arg-292 with lysine (39).

Here, in an attempt to change the substrate specificity of TPL from l-tyrosine to dicarboxylic substrates, we introduced an arginine residue in TPL in the same position that, as indicated by homology modeling, is occupied by Arg-292 in AspAT. Measurements of enzymic activities demonstrated indeed the conversion of TPL, a tyrosine β-lyase, to a dicarboxylic amino acid β-lyase. TPL R100T/V283R catalyzes the β-elimination reaction of l-aspartate at a 2-fold higher rate than that of l-tyrosine, the rate of β-elimination of l-aspartate being only 1 order of magnitude slower than that of β-elimination of l-tyrosine by the wild-type enzyme.

The dicarboxylic amino acids l-aspartate, l-glutamate, and l-2-amino-oacid are converted to pyruvate, ammonia, and the respective monocarboxylic acids, e.g. with l-aspartate as substrate, formation of formate was observed. The pathway of formate production from l-aspartate by β-elimination corresponds to that followed by the wild-type enzyme with its natural substrate l-tyrosine (Scheme 1). Upon formation of the quinonoid intermediate 2, the coenzyme donates electrons to the substrate resulting in the cleavage of the bond between Cβ and the nucleophilic leaving group thus producing the aminoacrylate coenzyme-substrate adduct 3. The cleavage of the aliphatic C-C bond very likely is facilitated by an active-site group which protonates the carbanion of the leaving carboxylic acid in a concerted fashion. Transimination of the aminoacrylate intermediate leads to the production of ammonium pyruvate 4, restoring the internal aldimine. The fact that no l-alanine was found in the reaction mixture together with the production of equimolar amounts of pyruvate and formate demonstrates that no β-decarboxylation occurred. Thus, β-elimination and slow transamination of l-aspartate (Table III) are the only reactions taking place. Analogous results have been obtained with l-glutamate and l-2-amino-oacid as substrates. It has to be noted that the known PLP-dependent carbon-carbon lyases acting on dicarboxylic amino acids are decarboxylases, e.g. glutamate decarboxylase (40) and aspartate β-decarboxylase (41, 42), that produce CO₂ and the cognate monocarboxylic amino acid. The reaction observed here reflects a newly generated catalytic activity for PLP-dependent enzymes toward dicarboxylic amino acids. TPL R100T/V283R and TPL R100T are the first Bₖ enzymes that catalyze the β-elimination reaction of these substrates.

The importance of the introduction of an arginine residue at position 283 for the recognition of dicarboxylic substrates is evident from a comparison of TPL R100T/V283R and TPL R100T. The single mutant TPL also catalyzes the β-elimination reaction of dicarboxylic substrates; however, the double mutant TPL R100T/V283R reacts faster with l-aspartate, and its Kₘ value for l-glutamate is lower (Table I). Moreover, the data agree with the hypothesis that Arg-100 is the positively charged group in the active site of TPL that interacts with the distal carboxylate group of l-aspartate or l-glutamate making them potential inhibitors but not substrates of wild-type TPL. This mode of inhibition of TPL by dicarboxylic acids has been proposed previously by Faleev et al. (34). Replacement of Arg-100 with threonine renders dicarboxylic substrates more flexible in the active site. The additional introduction of Arg-283 might stabilize the side chain carboxylate group in a more favorable position for reaction due to salt bridge-hydrogen bond interactions similar to those in AspAT (Fig. 4).

Recent studies (43) have demonstrated that Arg-381 in TPL is required for the recognition of l-tyrosine as substrate. We found that not only the R381I enzyme but also the triple mutant TPL R100T/V283R/R381I has no measurable activity toward both l-tyrosine and dicarboxylic substrates (data not shown).

Apparent dicarboxylic substrates adopt in the active site of TPL R100T/V283R a similar configuration as l-tyrosine in wild-type TPL and interact in a similar way with the critical residues of TPL that control reaction specificity. Even though the overall catalytic activity of TPL R100T/V283R toward l-tyrosine is decreased, its Kₘ value for this substrate is almost unchanged (Table I) suggesting that the side chain of Arg-283 contributes to the binding of the aromatic ring of l-tyrosine. Such interactions have been reported to apply in E. coli aromatic amino acid aminotransferase (44). How does the side chain of Arg-283 contribute to the recognition of l-tyrosine

**Table II**

**Stoichiometry of the β-elimination reaction of tyrosine phenol-lyase**

| R100T/V283R with l-aspartate | Products | Products |
|-----------------------------|----------|----------|
|                             | Pyruvate | Formate  |

**Table III**

**Kinetic parameters for side reactions of TPL wild-type and TPL R100T/V283R**

| Substrate | Activities | Kₘ’ | kₗ’ |
|-----------|------------|-----|-----|
| L-Aspartate | Transamination | 1.1 × 10⁻⁴ | 1.1 × 10⁻³ |
| L-Glutamate | Transamination | 1.3 × 10⁻⁴ | 1.5 × 10⁻³ |
| L-Serine | Transamination | 1.0 × 10⁻³ | 1.8 × 10⁻³ |
| L-Alanine | Transamination | 1.5 × 10⁻³ | 2.1 × 10⁻³ |
|           | Racemization | 0.03 | 8 × 10⁻³ |

*Measured at a single substrate concentration of 250 mM for l-aspartate, l-glutamate, l-serine, and of 100 mM for l-alanine.

1 From Ref. 10.
2 From Ref. 9.
The model of the “arginine switching” mechanism (45) assumes that the side chain of arginine moves out of the active site, when aromatic monocarboxylic substrates are bound. This model has been verified by x-ray crystallographic analysis of aspartate aminotransferase that has been engineered into a tyrosine aminotransferase (19, 46). Another model assumes that the guanidinium group of arginine directly links up with the aromatic ring of the bound substrates. This interaction may be energetically favorable (47).

The rate of the transamination half-reaction of dicarboxylic substrates is increased ten times by the two conjoint mutations. The values of $k_{cat}$ for the transamination of all the substrates studied here in the order of $10^{-3}$ s$^{-1}$. Apparently, the transamination reactions of these substrates depend on a rate-limiting step that follows the formation of the quinonoid intermediate 2. The $k_{cat}$ values of TPL R100T/V283R for transamination by and large coincide with those of the wild-type enzyme (Table III), indicating again that different active-site residues are important for the catalysis of $\beta$-elimination in transamination reactions.

In conclusion, the newly generated substrate specificity of TPL R100T/V283R agrees with previous studies in which the $B_5$ enzymes were changed without altering the reaction specificity by replacement of some critical active-site residues. The results are thus also consonant with evolutionary studies indicating that the $B_5$ enzymes originated from regio-specific catalysts, which first specialized for reaction site specificity and then for substrate specificity (2). The results of previous attempts to change the substrate specificity of aspartate aminotransferase by both site-directed mutagenesis (19) and forced molecular evolution (48) suggest that further improvement of the engineered dicarboxylic amino acid $\beta$-lyase, i.e. enhancement of its substrate binding affinity and catalytic efficacy, would require substitutions of numerous amino acid residues that do not participate in the active site.

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