Crystal Structures of Lysine-Preferred Racemases, the Non-Antibiotic Selectable Markers for Transgenic Plants

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Abstract

Lysine racemase, a pyridoxal 5'-phosphate (PLP)-dependent amino acid racemase that catalyzes the interconversion of lysine enantiomers, is valuable to serve as a novel non-antibiotic selectable marker in the generation of transgenic plants. Here, we have determined the first crystal structure of a lysine racemase (Lyr) from Proteus mirabilis BCR10725, which shows the highest activity toward lysine and weaker activity toward arginine. In addition, we establish the first broad-specificity amino acid racemase (Bar) structure from Pseudomonas putida DSM84, which presents not only the highest activity toward lysine but also remarkably broad substrate specificity. A complex structure of Bar-lysine is also established here. These structures demonstrate the similar fold of alanine racemase, which is a head-to-tail homodimer with each protomer containing an N-terminal (α/β)8 barrel and a C-terminal β-stranded domain. The active-site residues are located at the protomer interface that is a funnel-like cavity with two catalytic bases, one from each protomer, and the PLP binding site is at the bottom of this cavity. Structural comparisons, site-directed mutagenesis, kinetic, and modeling studies identify a conserved arginine and an adjacent conserved asparagine that fix the orientation of the PLP O3 atom in both structures and assist in the enzyme activity. Furthermore, side chains of two residues in α-helix 10 have been discovered to point toward the cavity and define the substrate specificity. Our results provide a structural foundation for the design of racemases with pre-determined substrate specificity and for the development of the non-antibiotic selection system in transgenic plants.

Introduction

Certain bacteria synthesize D-amino acids, e.g., D-Ala, D-Glu, D-Val, and D-Phe, for metabolic processes [1,2]. Interconversion of D- and L-amino acid enantiomers occurs not only in bacteria [3,4,5] but also in eukaryotes [6,7].

Two types of amino acid racemases are known: pyridoxal 5'-phosphate (PLP)-dependent [6] and PLP-independent enzymes [8]. Alanine racemase (Alr, E.C. 5.1.1.1), which catalyzes the interconversion of D- and L-Ala, is the best characterized of the prokaryotic and eukaryotic PLP-dependent enzymes. Several Alr crystal structures have been determined, and the structures are compact homodimers with each protomer containing an (α/β)8 barrel and a C-terminal domain having three β-sheets [9,10,11,12,13,14]. The dimeric interface contains the active-site cleft, which is formed by residues from the (α/β)8 barrel of one protomer and residues from the C-terminal domain of the other protomer. Structural and site-directed mutagenesis studies of Bacillus stearothermophilus Alr (BsAlr, PDB code: 1L6G) have identified two catalytic residues: Lys39 from one subunit and Tyr295 from the other subunit [13,15,16,17]. When alanine is absent, the C4A atom of PLP forms an aldime bond with the Lys39 Nε of BsAlr, whereas PLP forms an aldime bond with the amino nitrogen of the alanine substrate during catalysis [9,13].

A constricted entryway has been characterized in Mycobacterium tuberculosis Alr (MtAlr; PDB code: 1XFC) [18] that is believed to be the active site. The entryway is formed by three layers of residues (outer layer: Asp254, Lys352, and Ala351; middle layer: Arg316, Ile362, Arg396, and Asp401; inner layer: Tyr271, Tyr292, Tyr298, and Ala376) (prime symbol represents the residue from the other subunit of a dimer). After the entryway, PLP is found at the base of the cavity, and importantly, the strict Alr specificity for alanine is defined by the relatively small separation (~2.7 Å) between two innermost layer tyrosines: MtAlr, Tyr271 and Tyr292. Streptococcus pneumoniae Alr (SpAlr, PDB code: 3S46 [19]), Tyrr352, and Tyr355 [18].

In addition to Alrs, a PLP-dependent racemase is found to possess a broad amino-acid specificity [broad specificity amino acid racemase, Bar, E.C. 5.1.1.10 [20,21]]. Moreover, several microorganisms, including Proteus vulgaris [22], P. putida [23], Proteus spp., and Escherichia spp. [24], have lysine racemization activities, although the genes encoding lysine racemases and the lysine racemases themselves have not been isolated. Recently, fyr encoding a lysine racemase was obtained from a library con-
structured from the genetic material of garden soil microbes [25]. By screening a P. mirabilis BCRC10725 genomic library, a lyr was found that complemented a lysine-auxotrophic P. mirabilis mutant cultured on M9-containing agar supplemented with D-lysine [26].

Of note, the sequences of Bar and Lyr contain conserved lysine and tyrosine residues at positions equivalent to the catalytic residues of BsAlr (Bar: Lys, Tyr; Lyr: Lys, Tyr) [9,31], despite their otherwise limited sequence identity (20% between Bar and BsAlr; 24% between Lyr and BsAlr). Furthermore, the P. mirabilis BCRC10725 lyr sequence is identical with P. mirabilis ATCC29906 gene (NCBI accession number: ZP 03840526), which is annotated as a putative alanine racemase. According to a phylogenetic study that used Molecular Evolutionary Genetics Analysis, 24 prokaryotic amino acid racemases from 18 species have been classified into different groups: Group I contains Alrs from Gram-negative bacteria, Group II contains Alrs from Gram-positive bacteria, and Group III are non-Alr racemases, which have evolutionarily diverged from group I and II Alrs [26]. Lyr and Bar are therefore Group-III racemases. Expression and biochemical characterization of recombinant Lyr has shown that its preferred substrate is lysine [26]. Notably, Lyr is also active against arginine, whereas Bar racemizes arginine, lysine, methionine, serine, cysteine, leucine, and histidine [27], a useful feature for biotechnological applications.

It is interesting that lyr has been successfully used as a non-antibiotic selectable marker gene for plant transformation lately [28] since L-lysine is toxic to tobacco and Arabidopsis while D-lysine supports their growth [28]. Such a non-antibiotic selection has been successfully used as a non-antibiotic selectable marker gene for plant transformation lately [28].

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Results and Discussion

Bar and Lyr Structures

The final Lyr and Bar structures had R values of 22.0% (R\text{free} = 26.0%) and 15.8% (R\text{free} = 21.5%), respectively. Refinement statistics are given in Table 1. The asymmetric unit in the Bar crystal contains two molecules. With the exception of the positions for the first 25 residues, those of all other main-chain and side-chain atoms are well defined. Non-peptide electron density near Lys75 was fit with an aldimine bond between the Lys75 N\text{ε} and the PLP C4A. The average B-factor is 37.4 Å².

The Bar molecule contains two domains: an N-terminal (α/β) barrel, which contains PLP, and a β-stranded C-terminal domain (Figure 1A). Two Bar molecules associate to form an AB-type dimer, and their positions are related by a non-crystallographic two-fold axis (Figure 1B). The subunits have essentially the same fold (root mean square deviation (RMSD) = 0.219 Å for equivalent C\text{α} positions).

We next solved the Lyr structure by molecular replacement with the Bar structure as the template. One Lyr molecule is well defined. Non-peptide electron density near Lys74 was fit with an aldimine bond between the Lys74 N\text{ε} and the PLP C4A. The average B-factor is 37.4 Å². The average B-factor is 29.2 Å².

Molecular Lyr and Bar have the same domain structure, and PLP is found in an equivalent position in the two molecules (Figure 1C). Two Lyr molecules associate to form a dimer related by a two-fold crystallographic axis. In the Bar and Lyr dimers, the two protomers are arranged in a head-to-tail manner, stabilized by multiple interactions between N-terminal-domain residues in one monomer and C-terminal-domain residues in the other protomer (Figure 1B, D). The tertiary structures of the Lyr and Bar protomers are similar (RMSD = 0.89 Å for C\text{α} positions).

Structural Comparisons

When the Lyr or Bar structure was used as the query, the top-scoring proteins (Z-scores >35) returned by DALI were Alrs, even though the sequence identities for the Alrs and Lyr, and the Alrs and Bar are low (20–27% identity for Lyr; 23–31% identity for Bar). The tertiary folds of Lyr, Bar, and the Alrs are similar [29], and the protomers in each racemase are oriented head-to-tail.

Superposition of Lyr, Bar, SpAlr, BsAlr, and SlAlr confirmed their structural similarity (Figure 2A). The C-terminal domains superimpose relatively well (Q-scores within 0.68–0.95 for C\text{α} positions) [30]. However, the C\text{α} positions of a few regions in the N-terminal domains (corresponding to residues 108–121, 149–155, and 191–195 in Lyr) have lower structural similarities between our structures and Alrs (Q-scores within 0.45–0.60). Moreover, the C\text{α} positions in the ß3–ß10 loop between the N- and C-terminal domains have the greatest deviation (Q-scores within 0.38–0.45) and show that the hinge angles between the monomer domains are slightly different (Figure 2A), which are also discovered in Alr structures [14,19]. Lyr Lys and Bar Lys resides in the ß2–ß2 loop and bind PLP via an aldimine bond as has been found for the corresponding lysines and PLP in the Alrs (Figure 2B). The combined sequence and secondary structure alignments of Lyr, Bar, and the three Alrs demonstrate that this loop along with ß2, ß2, ß11, ß11–ß12, and ß12 share similar secondary structural elements (Figure 3). Additionally, the C\text{α} atoms in the sequences of these regions in Lyr and Bar superimpose very well with those in the Alrs (Q-scores within 0.85–0.95). Therefore, Lyr, Bar, and the Alrs probably originated from a common ancestor involved in racemization of amino acids for the synthesis of cell building blocks.

Racemase Active Sites

The Alr active-site cleft is located at the dimeric interface and contains the two conserved catalytic residues, a lysine from one subunit and a tyrosine from the other subunit (SpAlr: Lys and Tyr; BsAlr: Lys and Tyr; SlAlr: Lys and Tyr) [9,11,31]. Sequence alignment of Lyr, Bar, and the Alrs showed that the two catalytic residues are conserved in Lyr and Bar (Figure 2B). Furthermore, superpositioning the Lyr, Bar, SpAlr, BsAlr, and SlAlr structures showed that the two catalytic residues are situated at nearly identical positions within all five structures (Figure 1, 2, 3).

Most of the Alr PLP-binding residues are also conserved in Lyr and Bar (Table 2). Two conserved arginines (Lyr: Arg and Arg; Bar: Arg and Arg; SpAlr: Arg and Arg; BsAlr: Arg and Arg; SlAlr: Arg and Arg) in the (α/β) barrel interact with the pyridine N1 and the phenolic O (O3) of PLP, respectively, and orientate the PLP phenolic ring. A conserved lysine in SpAlr/BsAlr (Lys) can be carbamylated at its N\text{ε} (KCX) and interacts electrostatically with the guanidinium group of Arg (Figure 4). Instead, the side chain of the asparagine that is adjacent
to the corresponding arginine (Lyr: Asn\textsuperscript{174}; Bar: Asn\textsuperscript{175}) makes contacts with the arginine guanidinium moiety in Lyr and Bar, suggesting that these Asn/Arg interactions stabilize the orientation of PLP.

Table 1. X-ray Data and Refinement Statistics.

| Dataset                  | Lyr  | Bar  | Complex-Bar | SeMet-Bar (IMAD) |
|--------------------------|------|------|-------------|------------------|
| **Data collection and phasing** |      |      |             |                  |
| Beamline                 | SPring-8_SP44XU | NSRRC_BL13B1 | SPring-8_SP12B2 | NSRRC_BL13B1     |
| Space group              | C222\textsubscript{1} | C2 | C2 | C2 |
| Cell dimensions          |      |      |             |                  |
| a (Å)                    | 62.85 | 142.25 | 142.42 | 140.70 |
| b (Å)                    | 85.07 | 118.74 | 118.11 | 118.69 |
| c (Å)                    | 151.29 | 77.78 | 74.02 | 73.94 |
| Wavelength (Å)           | 1.0000 | 1.0000 | 1.0000 | 0.9797 |
| Resolution (Å)           | 30.00 – 1.74 | 30.00 – 2.45 | 20.00 – 3.10 | 30.00 – 2.10 |
| Highest resolution shell (Å) | 1.80 – 1.74 | 2.54 – 2.45 | 3.21 – 3.10 | 2.18 – 2.10 |
| Completeness (%)\textsuperscript{a} | 95.4 (100.0) | 99.7 (98.4) | 98.8 (93.4) | 97.9 (91.7) |
| Average I/σ(I)\textsuperscript{a} | 21.4 (3.5) | 15.4 (4.0) | 9.8 (3.3) | 16.8 (6.4) |
| No. of unique reflections | 39,932 | 46,692 | 21,648 | 69,330 |
| Redundancy\textsuperscript{a} | 5.1 (5.6) | 4.1 (4.0) | 4.2 (4.1) | 7.3 (6.6) |
| R\textsubscript{merge}\textsuperscript{a, b} | 6.2 (48.0) | 8.9 (46.1) | 15.3 (40.7) | 9.5 (24.6) |
| Overall figure of merit\textsuperscript{c} |          |          |          | 0.87 |
| Solvent content (%)      | 46.6 | 65.9 | 63.9 |
| **Refinement**           |      |      |             |                  |
| Resolution range (Å)     | 30.00 – 1.74 | 30.00 – 2.45 | 20.00 – 3.10 |
| Number of atoms          | Protein | 2890 | 5870 | 5890 |
|                         | Solvent | 224 | 381 | 95 |
| Average B-factor (Å\textsuperscript{2}) | 29.2 | 37.4 | 43.2 |
| R factor\textsuperscript{d} | 0.220 | 0.158 | 0.175 |
| R\textsubscript{free}\textsuperscript{d} | 0.260 | 0.215 | 0.219 |
| RMSD bond lengths (Å)\textsuperscript{f} | 0.020 | 0.017 | 0.017 |
| RMSD bond angles (°)\textsuperscript{f} | 1.477 | 1.469 | 1.376 |
| Estimated coordinate error (Å)\textsuperscript{f} | 0.092 | 0.268 | 0.271 |
| Ramachandran analysis (%)\textsuperscript{g} | 88.6 | 88.1 | 83.3 |
| Favored                  | 10.5 | 11.6 | 15.8 |
| Allowed                  | 0.9 | 0.3 | 0.9 |
| Generous                 | 0.0 | 0.0 | 0.0 |

\textsuperscript{a}Values in parentheses refer to statistics in the highest-resolution shell.
\textsuperscript{b}R\textsubscript{merge} = \Sigma ||F\textsubscript{obs}|| – ||F\textsubscript{calc}|| \Sigma ||F\textsubscript{obs}||.
\textsuperscript{c}Figure of merit = \|F\textsubscript{best}\|/\|F\|.
\textsuperscript{d}R = \Sigma ||F\textsubscript{obs}||\textsubscript{calc} – ||F\textsubscript{obs}|| \Sigma ||F\textsubscript{obs}||, where F\textsubscript{obs} and F\textsubscript{calc} are the observed and calculated structure-factor amplitudes, respectively.
\textsuperscript{e}R\textsubscript{free} was computed using 5% of the data assigned randomly.
\textsuperscript{f}Root mean square deviation.
\textsuperscript{g}Estimated standard uncertainties based on maximum likelihood.

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Lyr and Bar Substrate Binding Cavities

A funnel-like cavity (Figure S1) consisting of inner and outward layers is seen for Lyr or Bar (Figure 5), an arrangement that is also found in MtAlr [18]. An inner-layer tyrosine located in α-helix 10 (Tyr352 in SpAlr; Tyr354 in BsAlr) interacts with the PLP O3P—an interaction believed to be necessary for substrate specificity [32]. Together with the catalytic tyrosine, these two tyrosine residues (SpAlr: Tyr352 and Tyr265; BsAlr: Tyr354 and Tyr265), which are separated by ~2.7 Å, are strictly conserved and orient the alanine substrate and PLP properly. The α-helix 10 Tyr is not, however, present in Lyr and Bar, but is replaced by Thr391 in Lyr and Ala393 in Bar (Figure 4). Two water molecules contact the PLP O3P in Lyr and Bar, filling the space occupied by the tyrosine and PLP in SpAlr and BsAlr (Figure 4). Given the relatively larger space between Thr391 and PLP in Lyr, and Ala393 and PLP in Bar as compared with those in the Alrs, a substrate larger than an alanine may better fit the space.

For E. coli Alr, negatively and positively charged residues are found on the opposite sides of the surface of its cavity surface (Asp164, Glu165 and Arg259, Arg300) [33]. This array may help properly orientate the substrate for catalysis [33]. Superpositioning the corresponding residues in Lyr (Glu208, Asp209, Arg238, and Lys347), Bar (Glu208, Asp209, Arg238, and Lys347), SpAlr (Asp170, Glu171, Arg300, and Arg301), and BsAlr (Asp170, Glu171, Arg300, and Arg301) revealed that the charge distribution is similar in the four racemases (Figure 5, see also Figure S1), which supports the suggestion that these residues orient the substrate for catalysis.

Effects of Mutations on Enzymatic Activity

To evaluate the roles of Arg173, Asn174, and Thr191 in Lyr and Arg174, Asn175, and Ala192 in Bar (corresponding to Arg136, Leu137, and Tyr354 in BsAlr, respectively), we performed site-directed mutagenesis and characterized the effects of the mutations had on racemase activity. The conserved Arg173 in Lyr (Arg174 in Bar) was replaced with an alanine or a lysine. Asn174 in Lyr (Asn175 in Bar) was replaced with a leucine to mimic Leu137 in BsAlr. Thr191 in Lyr (Ala192 in Bar) was replaced with a tyrosine to mimic the conserved Alr tyrosines (Figure 4; Tyr354 in BsAlr and Tyr352 in SpAlr). Each mutant was expressed as a (His)6-tagged protein in E. coli and purified by Ni-affinity chromatography. CD analysis of each mutant revealed nearly identical profile to that of the wild-type enzyme (Figure S2). The racemase activity of each mutant toward L-Lys, L-Arg, and L-Ala was measured. R173A and R173K were inactive (Table 3) as were the corresponding Bar mutants R174A and R174K, supporting the importance of a conserved arginine at this position. Possibly, the Lyr Arg173 guanidinium group orients the PLP pyridine ring, to position the PLP-Tyr299 interaction for catalysis. The positive electrostatic field of the guanidinium group may also help lower the pKa value of the Tyr299 phenolic hydroxyl (pKa = 8.0–9.0) for effective catalysis.
A replacement of the neighboring asparagine with a leucine (N174L in Lyr and N175L in Bar) abolished racemization activity, which supports our hypothesis that this asparagine helps orient the PLP pyridine ring, revealing a comparable role as KCX found in Alr in conjunction with Arg173 in Lyr and Arg174 in Bar.

Mutation of the inner-layer Thr391 of Lyr to tyrosine, which is positioned similarly to the conserved tyrosines in Alrs (Tyr354 in BsAlr), reduced the activity of Lyr towards L-Lys to 53% (Table 3). The L-Lys \( k_{cat} \) value for this mutant was greater than that for Lyr, whereas the \( K_m \) value for the mutant with L-Lys as the substrate was 42% of Lyr (Table 4). Consequently, racemase activity for the mutant was significantly reduced (~50% residual activity, Table 3). A larger \( K_m \) value for L-Arg was also measured (Table 4), suggesting that the T391Y mutation might reduce the binding affinity for substrates with bulky side chains. In contrast, the \( k_{cat} \) values were comparable with L-Arg as the substrate, indicating that the transaldimination rate of PLP and L-Arg was unaffected by the mutation. No detectable activity was found when L-Ala was the substrate, implying that alanine still could not access the Lyr-T391Y active site.

The Bar A393Y mutant exhibited detectable, although reduced activity towards L-Lys (55% residual activity, Table 3). The \( k_{cat} \) value for the mutant with L-Lys as the substrate was reduced (44% residual activity, Table 4), and the \( K_m \) value increased 1.6-fold resulting in an overall reduction in activity. A393Y also was less activity toward L-Ala (25% residual activity), and the \( k_{cat} \) value towards L-Arg increased 2.1-fold [26]. Thus, this site may be also important for catalytic activity in Bar and Lyr. We generated the Lyr double mutant T391Y/S394Y, which exhibited significantly reduced specific activity towards L-Lys (6% residual activity) and toward L-Arg (0.9% residual activity) (Table 3). The \( k_{cat} \) value when L-Lys was the substrate decreased by 22-fold, whereas the \( K_m \) value decreased by only 1.6-fold (Table 4). Given these results, it is likely that the Thr391 and Ser394 side chains, which point toward the entryway, might help accommodate or orient the substrate to effect transaldimination with PLP. An even greater change in \( k_{cat} \) (28-fold) and in \( K_m \) (5-fold) was found when L-Arg was the substrate, suggesting that Thr391 and Ser394 in Lyr (Ala393 and Tyr396 in Bar) contribute to the binding and orientation of the substrate and therefore to catalytic activity. By considering specificity constants \((k_{cat}/K_m)_{Lys}/(k_{cat}/K_m)_{Arg}\) in comparing mutations of Lyr and Bar, T391Y has no significant change in substrate specificity (~2.9 fold) but T391Y/S394Y does enhance the Lys specificity over Arg (~15.0 fold). This implies that Ser394...
may be more important than Thr<sup>391</sup> for defining the substrate specificity in Lyr.

### Docked Lyr and Bar Models

We used an in silico docking method to generate the Lyr and Bar aldimine complexes: Lyr-PLP-D-Lys (Lyr-PLY), Lyr-PLP-D-Ala (Lyr-PDD), Bar-PLP-D-Lys (Bar-PLY), and Bar-PLP-D-Ala (Bar-PDD). The top docked poses with minimum RMSDs are shown in Table S2. In Lyr-PLY (Figure 6), a few residues closely interact with cross-linked D-Lys: (i) the catalytic residues Lys<sup>74</sup> and Tyr<sup>299</sup> contact the D-Lys amino nitrogen; (ii) Tyr<sup>318</sup> and Met<sup>347</sup> interact with the D-Lys carboxyl oxygens; and (iii) Thr<sup>391</sup> interacts with the D-Lys side-chain N<sub>f</sub>. Similar structural features are also found in Bar-PLY, except for a polar interaction between Ala<sup>393</sup> and D-Lys (Figure 6).

Conversely, Lyr-PDD and Bar-PDD (Figure 6) contain many fewer polar interactions between D-Ala and PLP. In Lyr-PDD, Lys<sup>74</sup> does not hydrogen bond with the amino nitrogen of D-Ala. Instead, D-Ala is positioned closer to Arg<sup>324</sup>, which is why L-Ala is not racemized by Lyr (Table 3). In Bar-PDD, D-Ala is closer to the PLP phosphate, which might also reduce its accessibility to the catalytic residue, Tyr<sup>301</sup>, and therefore decrease racemase activity.

### The Bar-Lys Complex Structure and Proposed Catalytic Mechanism

The complex-Bar structure was established (Figure 7A, B) by co-crystallizing Bar with L-lysine. The final Bar-L-lysine structure had R values of 17.5% (R<sub>free</sub> = 21.9%). The apo and liganded Bar structures share the same two-domain architecture and are both composed of the AB-type dimer. Superposition of the apo- and complex-form reveals a RMSD of 0.34 Å for the Cα atoms, demonstrating an overall identical conformation. Moreover, structural comparison of complex-Bar structure and Bar-PLY docking model shows that the reactive intermediates (PLP-lysine) are situated at the comparable position and surrounded by identical residues to form resembling hydrogen-bonding networks (Figure 7C), suggesting the feasibility of the docking approach utilized here.
### A

|                        | Lyr | Bar | BsAlr | SpAlr |
|------------------------|-----|-----|-------|-------|
| **Outward layer**      |     |     |       |       |
| Positively charged site| Lys$^{344'}$ | Lys$^{346'}$ | Arg$^{309'}$ | Arg$^{307'}$ |
|                        | Arg$^{324'}$ | Arg$^{326'}$ | Arg$^{290'}$ | Arg$^{288'}$ |
| Negatively charged site| Asp$^{209}$  | Asp$^{210}$  | Glu$^{172}$  | Glu$^{171}$  |
|                        | Glu$^{208}$  | Glu$^{209}$  | Asp$^{171}$  | Asp$^{170}$  |
|                        | Leu$^{389}$  | Leu$^{391}$  | Ile$^{352}$  | Ile$^{350}$  |
| **Inner layer**        |     |     |       |       |
| Catalyst               | Tyr$^{299'}$ | Tyr$^{301'}$ | Tyr$^{265'}$ | Tyr$^{263'}$ |
| Substrate binding site A| Thr$^{391}$  | Ala$^{393}$  | Tyr$^{354}$  | Tyr$^{352}$  |
|                        | Ser$^{394}$  | Ty$^{396}$   | Pro$^{357}$  | Val$^{355}$  |
| Substrate binding site B| Tyr$^{318'}$ | Tyr$^{320'}$ | Tyr$^{284'}$ | Tyr$^{282'}$ |
|                        | Met$^{347'}$ | Met$^{349'}$ | Met$^{312'}$ | Met$^{310'}$ |
| **The base**           |     |     |       |       |
| PLP-binding site       | Arg$^{173}$  | Arg$^{174}$  | Arg$^{136}$  | Arg$^{136}$  |
|                        | Asn$^{174}$  | Asn$^{175}$  | Leu$^{137}$  | Ile$^{137}$  |
|                        | Ala$^{165}$  | Ala$^{166}$  | Kcx$^{129}$  | Kcx$^{129}$  |

### B

[Insert figure showing the crystal structures of Lysine-Preferred Racemases]

[Map of amino acids and interactions with PLP-binding site and substrate binding sites A and B]
Two different mechanisms have been previously proposed for the reversible racemization of Alr enzymes: (1) the classical two-base mechanism [9] that describes the involvement of the quinonoid intermediate in the proton transfer between the two catalytic bases; and (2) a revised mechanism that proposes the substrate carboxylate group directly involved in the proton transfer based on the observation of a positively charged arginine (Arg219) situated by the pyridine nitrogen atom of PLP in structures of Alr-L-ala and Alr-D-ala [13].

Given the intimate contact between Arg219 and the pyridine nitrogen atom of PLP, it is not likely that L-ala and Alr-D-ala. Given the intimate contact between Arg219 and the pyridine nitrogen atom of PLP, it is not likely that a quinonoid intermediate proposed in the classical two-base mechanism could be formed. Instead, the substrate carboxylate group that is located in proximity to the two catalytic bases (Lys39 and Tyr265), proposed in the revised mechanism, is most plausible to serve as the critical moiety to mediate the proton transfer between the two catalytic bases.

The determined Bar-Lys complex structure also shows that the positive guanidium group of Arg261 (corresponding to Arg260 of Lyr) is close to the pyridine nitrogen atom of PLP (Figure 7C), hence may not favor the formation of the quinonoid intermediate proposed in the classical two-base mechanism. Moreover, the carboxylate group of L-lysine is situated at a position, allowing hydrogen-bonding interactions with both catalytic bases, respectively. Our docking models (Figure 6) are also in good agreement with the liganded Bar-Lys structure. These results together suggest the importance of the substrate carboxylate involved in the catalytic racemization for Lyr and Bar (Figure S3).

In conclusion, the determined structures of apo and L-Lys-liganded Bar present the internal and external aldimine linkages, respectively, as well as the anchored substrate in Bar. The apo Lyr structure has also been established within the internal aldimine linkage. Based on these structures, site-directed mutagenesis, kinetic, and modeling studies, a coordinated response between residues from α-helix 10 and those from the most peripheral region of the binding pocket (corresponding to Glu303 and Asp209, and Arg214 and Lys834 of Lyr) is essential for lysine-preferred racemases to ensure the substrate orientation, entry and specific binding, hence PLP-dependent catalysis. Our results also shed light on the evolution of PLP-dependent amino acid racemases that retain a converged funnel-like pocket having an outward layer that help anchor the substrate while a base platform for an efficient catalytic racemization. Structural and biochemical investigations in this study provide the key basis to figure out the enzymatic mechanism of Lyr and Bar, which helps to develop and improve the non-antibiotic selection system for transgenic plants.

### Materials and Methods

**Cloning, Expression, and Purification**

*P. mirabilis* BCR10725 *lyr* was PCR amplified from chromosomal DNA using gene specific primers (Table S1). Polymerase chain reaction (PCR) was performed with pfu DNA polymerase using a RoboCycler® GRADIENT 96 (Stratagene, La Jolla, CA, USA). Initial denaturation was performed at 95°C for 6 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 45 s, and primer extension at 72°C for 90 s. The amplified product was inserted into pET21a (Novagen, Inc., USA). Initial denaturation was performed at 95°C for 6 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 45 s, and primer extension at 72°C for 90 s. The amplified product was inserted into pET21a (Novagen, Inc., USA) to generate pET21a-*lyr*. *Escherichia coli* BL21(DE3) cells were transformed with pET21a-lyr, and expression of *lyr* was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration). An overnight culture of *E. coli* BL21(DE3) carrying pET-lyr or mutants was inoculated into 500 ml of LB

### Table 3. Racemization Activities toward L-Lys, L-Arg, or L-Ala by Wild-type Lyr and Bar, and Their Mutants.

| Enzymes            | L-Lys Specific activity (U/mg) | Ratio† | L-Arg Specific activity (U/mg) | Ratio | L-Ala Specific activity (U/mg) | Ratio |
|--------------------|-------------------------------|--------|-------------------------------|-------|-------------------------------|-------|
| Wild-type Lyr      | 2813 ± 98                     | 100%   | 568 ± 28                      | 20%   | ND                            | ND    |
| Lyr-R173A          | ND                            | ND     | ND                            | ND    | ND                            | ND    |
| Lyr-R173K          | ND                            | ND     | ND                            | ND    | ND                            | ND    |
| Lyr-N174L          | ND                            | ND     | ND                            | ND    | ND                            | ND    |
| Lyr-T391Y          | 1498 ± 66                     | 53%    | 527 ± 41                      | 19%   | ND                            | ND    |
| Lyr-T391Y/S394Y    | 170 ± 9                       | 6%     | 26 ± 6                        | 0.9%  | ND                            | ND    |
| Lyr-A165K/N174L/T391Y | ND                     | ND     | ND                            | ND    | ND                            | ND    |
| Wild-type Bar      | 2397 ± 97                     | 100%   | 1957 ± 70                     | 82%   | 192 ± 15                      | 8%    |
| Bar-R174A          | ND                            | ND     | ND                            | ND    | ND                            | ND    |
| Bar-R174K          | ND                            | ND     | ND                            | ND    | ND                            | ND    |
| Bar-N175L          | ND                            | ND     | ND                            | ND    | ND                            | ND    |
| Bar-A393Y          | 1316 ± 26                     | 55%    | 44 ± 2                        | 2%    | ND                            | ND    |
| Bar-A166K/N175L/A393Y | ND                     | ND     | ND                            | ND    | ND                            | ND    |

*The conversion yield of D-Lys from L-Lys was defined as 100% for wild-type Lyr and Bar. ND: Not detectable.

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medium containing ampicillin [100 μg/ml] and incubated at 37°C until an OD₆₀₀ of 0.7 was reached. Expression of the gene was induced by the addition of 0.5 mM IPTG and incubated at 17°C for 16 h. The cells were harvested by centrifugation at 10,000 × g for 10 min and resuspended in 50 ml of 100 mM potassium phosphate buffer (pH 8.0) containing 150 mM NaCl and disrupted by sonication. The cell debris was pelleted at 10,000 × g for 10 min and resuspended in 50 ml of 100 mM potassium phosphate buffer (pH 8.0). The purified Lyr was verified by SDS-PAGE analysis. Protein concentration was assayed by the Bradford method using bovine serum albumin as the standard [34].

**Site-Directed Mutagenesis**

pET21a-lyr and pET21a-bar served as the templates for overlap extension PCR amplification [35] of mutated genes using the primers listed in Table S1. Each amplified mutated gene was cloned into an XbaI–XhoI site in pET21a. The recombinant plasmids were individually introduced into E. coli Novablu or E. coli BL21(DE3). After isolating each plasmid, the presence of the desired mutation was confirmed by DNA sequencing.

**Circular Dichroism Analysis**

All circular dichroism (CD) measurements described here were made using 1.0 mm cells. Spectra were recorded at 25°C on AVIV 62A DS spectropolarimeter (Aviv Associates, Lakewood, NJ). The concentration of proteins used for wavelength scanning of CD measurement was 0.08 mg/ml. All spectra were recorded from 195 to 260 nm. Three spectra were recorded and averaged for each protein.

**Enzyme Assay**

Lyr and Bar racemase activities were measured using a 0.5 ml mixture of 0.5–1.0 U of purified enzyme, 100 mM CHES-NaOH (pH 9.0), 100 mM of an L-amino acid enantiomer, and 30 μM PLP. Each mixture was incubated at 50°C for 10 min, and the reactions were terminated by heating at 100°C for 10 min. The chiralities of the amino acids were characterized by high-performance liquid chromatography using a Chirobiotic T column (4.0×150 mm, Advanced Separation Technologies, USA) at a flow rate of 0.5 ml/min. The mobile phase was 50% ethanol/50% NaH₂PO₄ (pH 4.5). The detection wavelength was 210 nm. One unit of racemase activity was defined as the amount of enzyme that produced 1 μ mole of the amino acid enantiomeric product from the enantiomeric reagent per min.

A steady-state kinetics study of the purified enzymes was performed at 50°C with a reaction mixture that contained 100 mM CHES-NaOH (pH 9.0), 30 μM PLP, and an amino acid enantiomer (concentration, 10–150 mM). The Km and kcat values were determined by plotting the initial velocity as a function of substrate concentration and fitting the plots to the Michaelis-Menten equation.

**Crystallization**

Crystallization was performed by the sitting-drop vapor-diffusion method at 20°C. Equal volumes of a protein sample and the reservoir solution were mixed. Initial crystallization screening was automated using a robot Oryx8 (Douglas Instruments, UK) and the reagents of seven sets of crystallization kits: Crystal Screen I and II kits (Hampton Research), Index kit (Hampton Research), Clear Strategy Screen I and II kits (Molecular Dimension), Wizard kit (Emerald), and JB Screen classic HTS I and II kits (Jena Bioscience).

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**Table 4.** Kinetic Parameters for Wild-type Lyr and Bar, and Their Mutants.

|      | L-Lys |       |       |       | L-Arg |       |       |       | Specificity constants |
|------|-------|-------|-------|-------|-------|-------|-------|-------|-----------------------|
|      | kcat (min⁻¹) | Km (mM) | kcat/Km (A) |       | kcat (min⁻¹) | Km (mM) | kcat/Km (A) |       | (A/B) |
| Wild-type Lyr | 3326±68 | 21.8±0.5 | 152.6 |       | 650±35 | 149±1.0 | 43.6 |       | 3.5 |
| Lyr-T391Y | 1384±148 | 37.6±5.3 | 36.8 |       | 610±12 | 47.7±1.7 | 12.8 |       | 2.9 |
| Lyr-T391Y/S394Y | 154±11 | 34.3±4.1 | 4.5 |       | 23±3 | 75.5±6.2 | 0.3 |       | 15.0 |
| Wild-type Bar | 3545±377 | 25.4±0.6 | 139.6 |       | 2228±14 | 143.5±1.2 | 153.7 |       | 0.9 |
| Bar-A393Y | 1456±131 | 33.9±3.5 | 42.9 |       | – | – | – |       | – |

The random microseed matrix screening method [36] was used to obtain crystals suitable for diffraction. The volumes of the protein sample, reservoir solution, and microseed solution were 0.5 μl, 0.4 μl, and 0.1 μl, respectively. Optimized crystals were grown in 0.1 M sodium acetate (pH 4.6), 0.05 M lithium chloride, 29% (w/v) PEG 8000. The crystal diffracted to 1.74 Å, belonged to space group C2221, and had unit cell dimensions of a = 142.25 Å, b = 118.94 Å, c = 151.29 Å. The asymmetric unit contained one molecule.

Crystals of Lyr (2.5 mg/ml) were grown in 0.1 M sodium acetate (pH 4.5), 0.2 M lithium sulfate, 30% (w/v) polyethylene glycol (PEG) 8000. Optimized crystals used for diffraction were grown in 0.1 M sodium acetate (pH 4.6), 0.05 M lithium chloride, 29% (w/v) PEG 8000. The crystal diffracted to 1.74 Å, belonged to space group C2221, and had unit cell dimensions of a = 62.85 Å, b = 85.07 Å, c = 151.29 Å. The asymmetric unit contained one molecule.

Crystals of Bar (4.7 mg/ml in 10% (v/v) glycerol) were grown in 0.1 M Tris-HCl (pH 8.5), 2.0 M ammonium sulfate. The crystal diffracted to 2.45 Å, belonged to space group C22, and had unit cell dimensions of a = 142.25 Å, b = 118.74 Å, c = 77.78 Å. The asymmetric unit contained two molecules.

Crystals of selenomethionine-derivative Bar (SeMet-Bar) (8.1 mg/ml in 10% (v/v) glycerol) were initially grown in 0.1 M Tris-HCl (pH 8.5), 0.2 M sodium acetate, 25% (v/v) PEG 3350. The random microseed matrix screening method [36] was used to obtain crystals suitable for diffraction. The volumes of the protein sample, reservoir solution, and microseed solution were 0.5 μl, 0.4 μl, and 0.1 μl, respectively. Optimized crystals were grown in 0.1 M Tris-HCl (pH 8.5), 0.2 M lithium sulfate, 1.26 M ammonium sulfate. The SeMet-Bar crystal belonged to space group P2₁, and had unit cell dimensions of a = 140.89 Å, b = 118.94 Å, c = 74.17 Å. The asymmetric unit contained two molecules.

Crystals of the complex Bar protein (6.1 mg/ml within 4 mM L-lysine) were obtained from the condition using 0.1 M bicine...
Figure 6. Protein-ligand interactions in crystal structures and docked models (See also Figure S4 and Table S2, S3, S4, S5). Residues of Lyr and Bar are colored green and magenta, respectively. The docked substrates presented as the external aldimine form inside Lyr and Bar are colored gray. The hydrogen bonding is labeled by red dashed lines. Oxygen, nitrogen, and phosphate atoms are colored red, blue, and orange, respectively.

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Figure 7. Bar-PLP-L-lysine complex structure. (A) The dimeric Bar is presented as cartoon presentation (chain A, wheat and chain B, orange). PLP and L-lysine are drawn as stick models. (B) The omit Fo-Fc electron density map shows PLP, substrate (L-lysine), and the external aldimine linkage, contoured at the 1.0-σ level. (C) Structural comparison of the binding pockets for apo Bar (magenta), L-lysine-ligated Bar (wheat) structures and Bar-PLY (gray) docking model. PLP, substrate L-Lys and catalytic K75 are shown as ball-and-stick models, whereas catalytic Y301′ from the other protomer is drawn as heavy sticks. The rest of surrounding residues are shown as thin sticks. Oxygen, nitrogen, and phosphate atoms are colored in red, blue, and orange, respectively.

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and Bar structures were assessed by PROCHECK [44]. The Bar-complex FMAC5 and coupled to ARP/wARP. The Lyr structure was constructed by the molecular replacement program in PHENIX with the Bar protomer (50% identity) as the template. Crystallographic refinement used the maximum-likelihood target function module in REFMAC5 [39,40]. The apo- and complex-form Bar structures were constructed by MOLREP [39,40,41] and were refined using REFMAC5 coupled with ARP/wARP [42], which automatically added water molecules.

The Lyr structure was constructed by the molecular replacement program in PHENIX with the Bar protomer (50% identity) as the template. Crystallographic refinement used the maximum-likelihood target function as implemented in REFMAC5 and coupled to ARP/wARP.

For the Lyr, Bar, complex-Bar and SeMet-Bar structures, 2Fo−Fc and Fo−Fc maps were produced and inspected after each automated refinement cycle to manually refine the structures with the aid of COOT [43]. The validities of the Lyr, Bar and complex-Bar structures were assessed by PROCHECK [44].

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/pdb/) under the accession codes 4DZA (Lyr), 4DJY (Bar), and 4FS9 (Bar-L-lysine).

Structural and Sequence Comparisons

The Lyr and Bar structures were compared with all protein structures in the DALI server (http://ekhidna.biocenter.helsinki.fi/dali_server/). The structures of Lyr, Bar, complex-Bar, BsAlr [13], SpAlr [19], Streptomyces lavendulae Alr (SlAlr, PDB code: 1VFS; [12]), and E. coli diaminopimelate decarboxylase-lysine complex (PDB code: 1KO0) were superimposed by LSQMAN in O [45] to determine the pair-wise RMSD of the Cα atom positions. Q-score, a common measure to present the three-dimensional similarity, was applied to structural comparisons and ranged from 0, where no similarity exists, to 1 where structures are identical [30,46,47]. ESPript was used for the combined sequence and secondary structure alignments and figure preparation [48]. PyMol (http://www.pymol.org) was used to prepare the figures containing structures.

Structural Modeling

Discovery Studio v3.0 (Accelrys Inc., USA) was used to prepare, energy minimize, and refine the Lyr and Bar structures prior to docking D-Ala and D-Lys into Bar and Lyr, respectively, and for molecular dynamics. The default parameters of ChiRotor were used [49] for optimizing both protein side-chain conformations. Energies of the protein models were further minimized using CHARMM [50].

To prepare PLP-D-alanine (PDD) and PLP-D-lysine (PLY) models, D-Ala and D-Lys coordinates were extracted from the BsAlr (PDB code: 1L6G) [13] and E. coli D-lysine-diaminopimelate decarboxylase-lysine complex (PDB code: 1KO0), respectively. LibDock [51] was used in conjunction with its default settings to generate conformations for ligand-containing Lyr and Bar. Hot spots were then aligned to select docked poses, which were then in situ minimized by Discovery Studio 3.0 “Conjugate Gradient” [52] to improve the quality of the conformations. All poses were scored, and the top 100-scoring poses were retained for subsequent analyses. Approximately 20% of the poses were retained from the generated 255 poses for each model (Bar: Bar-PDD, Bar-PLY; Lyr: Lyr-PDD, Lyr-PLY). Two additional scoring functions, Potential of Mean Force [53] and LigScore2 scoring [54,55], were used to compensate for an unfair penalty imposed on the binding-affinity measurement, which resulted because hydrogen atoms were eliminated during LibDock docking. The consensus score for the docked ligands in each protein-lysine model was calculated. Then, CDOCKER [56] with CHARMM forcefield was used to further refine the docked models.

The phosphate moiety in each cognate ligand (PDD or PLY) was fixed in place because its position was superimposable with those in the Alr crystal structures that were complexed with PLP (PDB codes: 1SFT [9], 1BD0 [10], 1L6G [13], 1VFS [12]) as a consequence of an extensive hydrogen-bonding network. When the RMSD of the phosphate in a docked model deviated by ≤1 Å with that of the reference pose (Table S2, S3, S4, S5), the docked model was selected for further study. According to the established mechanism of BsAlr (PDB code: 1L6G) [13]), both catalysts (Lys39 and Tyr265) were close to the Cα atom of the alanine substrate within hydrogen bonding distances and responsible for the racemization reaction. Hence, the distances between the atom Cα of docking ligands and reactive atoms of two conserved catalysts (Lys:N and Tyr:O) of Lyr and Bar will be constrained to the extensive hydrogen bonding distance (<4 Å) (Table S2–S5).

To estimate the practicability of the proposed docking procedure, we attempted to self-dock the co-crystal structures complexed with PLP-D-alanine (PDB code: 1L6G) [13] and PLP-D-lysine (PDB code: 1KO0). Top five docked poses with minimum RMSD ≤1 Å were derived. On average, the RMSD of self-docked poses for PLP-D-alanine and PLP-D-lysine are 0.65 Å and 0.84 Å respectively (Figure S4). This suggests that the proposed docking approach is viable.

Supporting Information

Figure S1 Related to Figure 5: Top-view and side-view of the binding cavities of Lyr, Bar, and Alrs. The views are those obtained by superpositioning the structures of Lyr (green), Bar (magenta), SpAlr (yellow) and BsAlr (cyan). Crucial residues at the substrate entryway are displayed as stick models. PLP-lysine of Lyr, Bar, and SpAlr as well as PLP-D-lysine plus K39 of BsAlr, (pH 9.0), 0.1 M sodium chloride, and 20% (w/v) polyethylene glycol monomethyl ether (PEG-MME) 550. The Bar-complex crystal diffracted to 3.10 Å and belonged to space group C2 with the unit cell dimensions a = 142.42, b = 116.11, c = 74.02 Å. There were also two molecules per asymmetric unit.
respectively, are drawn as ball-and-stick models. Spheres of the conserves tyrosine catalyzes are colored as magenta. Spheres of positively charged, negatively charged, polar, and non-polar residues are colored as blue, red, pink, and yellow, respectively. Oxygen, nitrogen, and phosphate atoms are colored red, blue, and orange, respectively.

**Figure S2** CD spectra of recombinant proteins. (A) The wavelength scanning results of Lyr wild-type and mutant proteins. (B) The wavelength scanning results of Bar wild-type and mutant proteins.

**Figure S3** Scheme of the proposed catalytic mechanism of Lyr and Bar. The substrate, lysine, is colored in red. Catalytic bases are shown as Lys\(^75\) and Tyr\(^{301}\) of Bar (corresponding to Lys\(^74\) and Tyr\(^{299}\) of Lyr).

**Table S1** Oligonucleotide primers used in this study.

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