Supplementary Material (ESI) for Chemical Communications

ELECTRONIC SUPPORTING INFORMATION

Engineering of an enantioselective tyrosine aminomutase by mutation of a single active site residue in phenylalanine aminomutase

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Experimental Section

Chemicals

(*S*)-*α*-phenylalanine was obtained from Sigma-Aldrich-Fluka. (*S*)-*α*-tyrosine was purchased from J. T. Baker Chemicals. (*S*)-*β*-phenylalanine, (*R*)-*α*-phenylalanine and (*R*)-*β*-tyrosine were synthesized by Peptech. corp. (*R*,*S*)-*β*-tyrosine was from Innochemie GmbH.

Construction of the PAM mutants

The C107H/F/S mutants of PAM were generated using the mega-primer method and the coding sequence for PAM in plasmid pBAD-His-PAM as the template. In the first stage of PCR, the mega-primer was made by using the primer C107H: 5′-CCTGATCCGTCATCTGCTGGCCGGCGTGTTCACC-3′, C107F: 5′-CCTGATCCGTTTTCTGCTGGCCGGCGTGTTCACC-3′ or C107S: 5′-CCTGATCCGTAGCCTGCTGGCCGGCGTGTTCACC-3′, with the mutated codon underlined, and the primer 5′-AACGGTAGACGCAACTGCAGTAG-3′ (designated *Pst*I reverse primer). In the second stage of PCR, the mega-primer was used in combination with primer 5′-CGCGCGGCAGCCATATGGGTTTC-3′ (designated *Nde*I forward primer) to amplify the full length mutant gene. When the second PCR

was finished, 10 units of *Dpn*I was added directly to the PCR mixture and the mixture was incubated at 37 ^{o}C for 2 h to remove the template DNA. The final PCR product was purified, digested with *Nde*I and *Pst*I, and ligated into the *Nde*I/*Pst*I treated pBAD-His vector. The ligation mixtures were transformed into competent *E. coli* TOP10 cells and transformants were plated on LB plates with ampicillin. All mutant constructs were confirmed by sequencing the region between the *Nde*I and *Pst*I sites. All primers were supplied by Sigma-Aldrich.

Expression and purification of the PAM mutants

The PAM mutants were produced in *E. coli* TOP10 using the pBAD expression system (Invitrogen). Fresh TOP10 cells containing the appropriate expression plasmid were used to inoculate 10 mL of LB medium containing 50 μg/ml ampicillin (LB/Amp medium). After overnight growth at 37 °C, 10 mL of the culture was used to inoculate 1 L of LB/Amp medium. Cultures were grown to an OD_{600} of 0.5 at 37 °C with vigorous shaking and then induced with arabinose (0.002% (w/v) final concentration). Incubation was continued for 20 h at 17 °C. Cells were harvested by centrifuging at 5000 g for 20 min, washed with 50 ml of buffer A (20 mM Tris-HCl buffer, pH 8.0, 20 mM imidazole, 0.5 M NaCl) and suspended in 50 ml of the same buffer. Cells were disrupted by sonication, after which unbroken cells and debris were removed by centrifugation (15,000 g, 60 min). All steps were performed at 4 ^{o}C. PAM was purified by Ni-based immobilized metal affinity chromatography column using an AKTA system (Amersham). After washing with 20 mM and 60 mM imidazole, the bound protein was eluted with 200 mM imidazole. Fractions containing PAM were desalted and concentrated to 8 mg/ml in storage buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 25% glycerol, pH 8.0). The purified enzyme was flash-frozen in liquid nitrogen and stored at -80 ^{o}C until further use. The purity of the protein was analyzed by SDS-PAGE and the protein concentration was determined by the Bradford assay.

Determination of aminomutase activity

The mutase activity of PAM mutants was determined by monitoring the production of *β*-amino acids upon incubation with the corresponding *α*-amino acids. Incubation mixtures contained *α*-phenylalanine or *α*-tyrosine (0.002 mM to 0.2 mM), Tris-Cl buffer (50 mM, pH 8.0), and purified PAM mutants (0.08 mg). At different times, samples (34 µl) were taken from the reaction mixture and quenched by adding HCl (2 M, 18 µl) with cooling on ice for 5 min. The pH was re-adjusted to 8.0 by adding small aliquots of an aqueous KOH solution. The reaction mixture was directly loaded onto the HPLC system.

A Thermo aquasil C18 5u column (5 µm, 250 mm x 4.6 mm) was used for analytical separation of *α*- and *β*-phenylalanine/tyrosine. The solvents were phosphate buffer (50 mM, pH 3.0) (eluent A) and HPLC pure CH_3CN (eluent B). The flow rate was 1 ml/min. The elution gradient was formed as follows: start with 98:2 for 15 min, in 10 min from 98: 2 to 50: 50, and from 50: 50 to 98:2 from 25 min to 40 min. The analyses were carried out at 20 ^{o}C, with detection at 210 nm. The initial rates were plotted against the substrate concentrations fitted to the Michaelis-Menten equation to obtain the kinetic parameters.

Product identification of ammonia addition reaction

Reaction mixtures contained 4-hydroxycinnamic acid (3 mM), ammonia (6 M, pH 10), and PAM-C107S mutant (0.3 mg/ml). Reactions were initiated by the addition of enzyme, incubated at 30 ^{o}C, and quenched by heating for 5 min at 99 ^{o}C. The reaction mixtures were directly loaded on A Thermo aquasil C18 5u column (5 µm, 250 mm x 4.6 mm). HPLC analysis was identical to the method used for determination of aminomutase activity.

Analysis of the stereochemistry of β-tyrosine products by chiral HPLC.

The stereochemistry of PAM mutants was determined by monitoring the production of *β*-tyrosine upon incubation with *α*-tyrosine. Incubation mixtures contained 0.2 mM *α*-tyrosine, 50 mM Tris-Cl buffer (pH 8.0), and 0.08 mg of purified PAM mutant. Analysis was carried out on a Crownpak CR (+) (4 mm x 150 mm)

column. Compounds were eluted isocratically with $HClO_4$ in 15% MeOH (pH 3.0) with UV detection at 210 nm. The flow rate was 0.3 ml/min and the temperature of the column was maintained at -5 ^{o}C. The retention time for (*R*)-*β*-tyrosine, (*S*)-*α*-tyrosine and (*S*)-*β*-tyrosine are 39 min, 44 min and 50 min respectively.