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Enzymatic characterization and crystal structure of biosynthetic alanine racemase from *Pseudomonas aeruginosa* PAO1

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ABSTRACT

Alanine racemase is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that reversibly catalyzes the conversion of L-alanine to D-alanine. D-alanine is an essential constituent in many prokaryotic cell structures. Inhibition of alanine racemase is lethal to prokaryotes, creating an attractive target for designing antibacterial drugs. Here we report the crystal structure of biosynthetic alanine racemase (Alr) from a pathogenic bacteria *Pseudomonas aeruginosa* PAO1. Structural studies showed that *P. aeruginosa* Alr (PaAlr) adopts a conserved homodimer structure. A guest substrate D-lysine was observed in the active site and refined to dual-conformation. Two buffer ions, malonate and acetate, were bound in the proximity to D-lysine. Biochemical characterization revealed the optimal reaction conditions for PaAlr.

1. Introduction

L-amino acids are the building blocks of proteins for life. D-amino acids are also fundamental in microbial physiology [1]. D-amino acids (mainly D-ala and D-glu) produced by microbial amino acid racemases have been described as relevant constituents in many prokaryotic cell structures [2].

Alanine racemase (Alr) is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that catalyzes the conversion of L-alanine to D-alanine [3],which is an essential component in the biosynthesis of the peptidoglycan layer of the cell walls [3,4]. Alanine racemase is ubiquitous among bacteria but is generally absent in human and rare in eukaryotes [2]. Lack of Alr activity is normally lethal to these prokaryotic organisms in the absence of an exogenous source of D-Ala [5,6], thus making the enzyme an attractive target for antibacterial drugs [7].

Whereas some organisms contain only one alanine racemase [8–10], two kinds of alanine racemases have been identified in some gram-negative bacteria [11]: the *Alr*-encoded alanine racemase, which is constitutive and used for D-Ala biosynthesis; and the *DadX*-encoded alanine racemase, which is inducible and used for the catabolism of D-Ala [2,3,11–14].

Pseudomonas aeruginosa is one of the most frequent and severe causes of hospital–acquired infections, particularly affecting immunocompromised (especially neutropenic) and Intensive Care Unit (ICU) patients [15]. Two alanine racemases were identified from *P. aeruginosa* PAO1: biosynthetic (PaAlr) and catabolic (PaDadX) [16]. These enzymes show an approximately 50% sequence identity to the alanine racemases of *Escherichia coli*. In-solution studies indicated that alanine racemases were in a dynamic equilibrium between monomeric and dimeric form, with the homodimer being the catalytically active form [17].

Crystal structures of Alrs from many species, such as *Geobacillus stearothermophilus* [18], *P. aeruginosa* [19] and *E. coli* [20]. have been reported. These studies confirmed that Alr is a homodimer, with each monomer containing two folded domains. The N-terminal domain is an α/β barrel, and the C-terminal domain is composed of β-strands. The two active sites in the homodimer are formed by residues from both the N- and C-terminal domains. PLP is covalently linked to a conserved Lys in the N-terminal domain.

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Structural [21–23] and enzymatic activity [24,25] studies revealed a two-base catalytic mechanism for reversible catalysis, involving key catalytic residues Lys39 from one subunit and Tyr265′ from the other.

LeMagueres et al. [19] reported the crystal structure of PaDadX, whereas the structure of PaAlr is still unknown. In this study, we determined the crystal structure of PaAlr and revealed a conserved quaternary structure of a homodimer. Sequence alignment and structure comparison showed some strictly conserved residues. A guest substrate D-lysine was observed in the active site and refined to dual-conformation. Two buffer ions, malonate and acetate, were found to bind in proximity to D-lysine. The enzymatic activities tested in various conditions provided the optimal reaction conditions and strict substrate specificity.

2. Materials and methods

2.1. Enzymatic assay

The enzymatic activity of PaAlr was measured as described previously with slight modifications [26]. The standard racemization mixture was composed of 20 mM Britton-Robinson buffer (pH 10.0), 10 μM Pyridoxal 5′-phosphate (PLP), 50 mM L-alanine and purified protein for a final volume of 200 μL. Bovine serum albumin (BSA) instead of enzyme was used as the negative control. The enzymatic reaction was performed at 313K for 10 min. D-amino acid oxidase and peroxidase were used to measure the D-forms of amino acids as described [26,27]. The D-amino acid oxidase reaction was performed at a volume of 200 μL with 200 mM Tris-hydrochloric acid (HCl) pH 8.0, 0.1 mg mL^{-1} 4-aminoantipyrine, 0.1 mg mL^{-1} TOOS, 2 units (U) peroxidase (Horseradish, Sigma) and 0.1 U D-amino acid oxidase (Porcine kidney, Sigma). The absorbance at 550 nm was measured using Epoch Microplate Spectrophotometer (BioTek, USA). One unit (U) of the enzyme was defined as the amount of enzyme to catalyze the formation of 1 μmol of D- or L-alanine from either enantiomer per minute.

2.2. Biochemical characterization of PaAlr

The optimal pH and buffer of the purified recombinant PaAlr

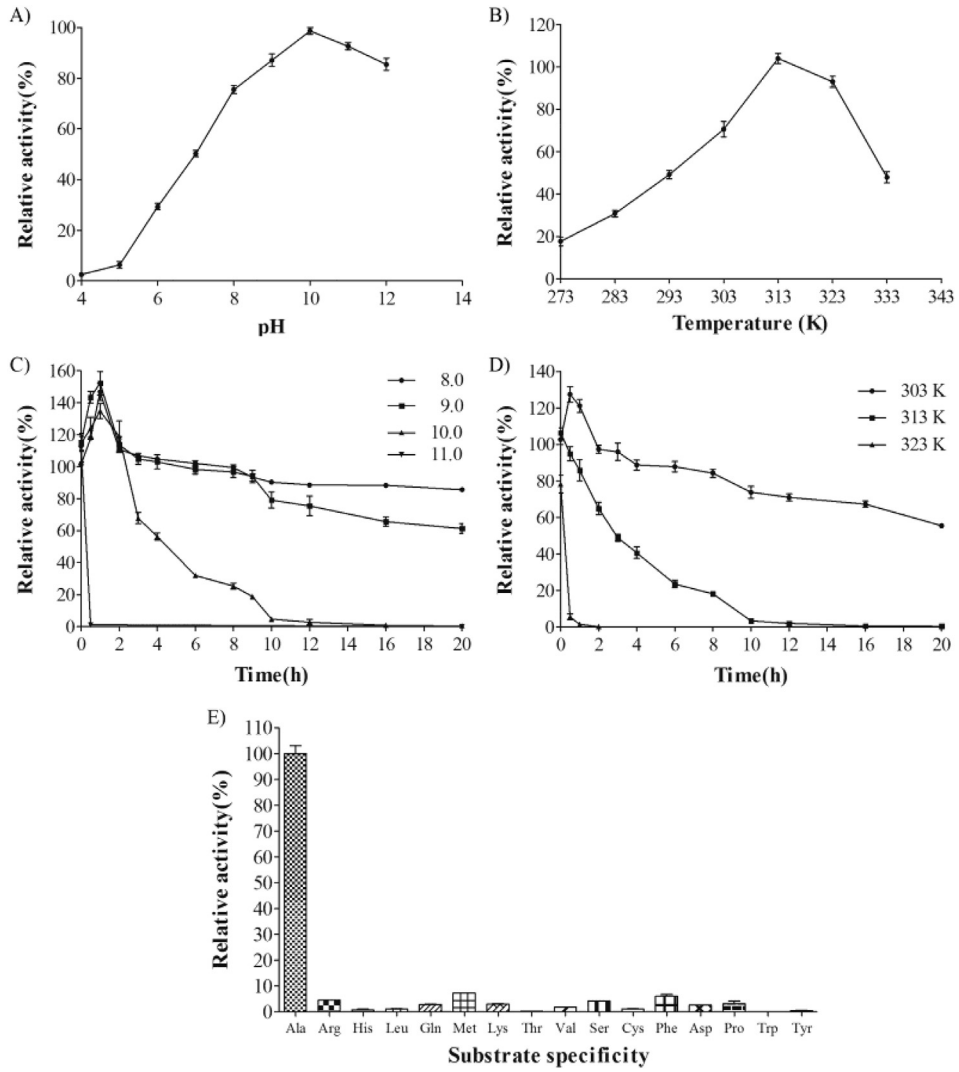

Fig. 1. The enzymatic properties of *Pseudomonas aeruginosa* PAO1 biosynthetic alanine racemase (PaAlr). **(A)** Optimal pH of PaAlr at 313 K in 20 mM Britton-Robinson buffer (pH 4.0–12.0). **(B)** Optimal temperature of PaAlr from 273 K to 333 K (20 mM Britton-Robinson buffer, pH 10.0). **(C)** pH-dependent stability at 313 K for 0–20 h. Cycle (●): pH 8.0; square (■): pH 9.0; triangle (▲): pH 10.0; and inverted triangles (▼): pH 11.0. **(D)** Thermostability of PaAlr in pH 10.0 buffer for 20 h at 303 K (●), 313 K (■), and 323 K (▲). **(E)** Substrate specificity. The relative activity of PaAlr for various L-amino acids was determined at 313 K and pH 10.0. The data were presented as the mean values ± SD from three independent enzymatic assays with triplicate determinations.

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