Enzyme and Microbial Technology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Research Paper

A conserved residue of L-alanine dehydrogenase from *Bacillus pseudofirmus*, Lys-73, participates in the catalytic reaction through hydrogen bonding

Guangzheng He[a,1], Shujing Xu[a,b,1], Shanshan Wang[a], Qing Zhang[c], Dong Liu[a], Yuling Chen[a], Jiansong Ju[a,*], Baohua Zhao[a,*]

[a] College of Life Science, Hebei Normal University, Shijiazhuang, Hebei, PR China
[b] College of Tourism, Hebei Normal University, Shijiazhuang, Hebei, PR China
[c] Haibin College, Beijing Jiaotong University, Cangzhou, Hebei, PR China

ARTICLE INFO

Keywords:
L-Alanine dehydrogenase
Catalytic activity
Hydrogen-bonding
Mutation

ABSTRACT

A multiple protein sequence alignment of L-alanine dehydrogenases from different bacterial species revealed that five highly conserved amino acid residues Arg-15, Lys-73, Lys-75, His-96 and Asp-269 are potential catalytic residues of L-alanine dehydrogenase from *Bacillus pseudofirmus* OF4. In this study, recombinant OF4Ald and its mutants of five conserved residues were constructed, expressed in *Escherichia coli*, purified by His_6-tag affinity column and gel filtration chromatography, structure homology modeling, and characterized. The purified protein OF4Ald displayed high specificity to L-alanine (15 U mg^{-1}) with an optimal temperature and pH of 40 °C and 10.5, respectively. Enzymatic assay and activity staining in native gels showed that mutations at four conserved residue Arg-15, Lys-75, His-96 and Asp-269 (except residue Lys-73) resulted in a complete loss in enzymatic activity, which signified that these predicted active sites are indispensable for OF4Ald activity. In contrast, the mutant K73A resulted in 6-fold improvement in k_{cat}/K_m towards L-alanine as compared to the wild type protein. Further research of the residue Lys-73 substituted by various amino acids and structural modeling revealed that residue Lys-73 might be involved in the catalytic reaction of the enzyme by influencing the enzyme-substrate binding through the hydrogen-bonding interaction with conserved residue Lys-75.

1. Introduction

L-Alanine dehydrogenase (AlaDH; L-alanine: NAD^+ oxidoreductase, EC 1.4.1.1) catalyzes the reversible, NAD^+-dependent oxidative deamination of L-alanine to pyruvate and ammonia [1,2]. This enzyme plays an important role in the carbon and nitrogen metabolism of microorganisms and is a key factor in the assimilation of L-alanine as an energy source through the tricarboxylic acid cycle [3–5]. Previous studies reported that L-alanine dehydrogenase is the key enzyme for the de novo synthesis of alanine in *Rhizobium leguminosarum* [4,6]. AlaDH is also involved in the generation of energy during sporulation process [6–8]. AlaDHs have been cloned and characterized from different bacterial species, such as *Archaeoglobus fulgidus* [9], *R. leguminosarum* [4], *Mycobacterium tuberculosis* [10], and *Thielaviopsis paradoxa* [11].

At present, a few crystal structures of alanine dehydrogenases and its complexes with NAD^+ or pyruvate from several microorganisms are available in Protein Data Bank (PDB). Based on the catalytic mechanism and crystal structures analysis of the alanine dehydrogenase from *Phormidium lapideum* (PDB ID: 1PJB) [1], *M. tuberculosis* (PDB ID: 2VHY) [12] and *Thermus thermophilus* (PDB ID: 2EEZ), four residues Arg-15, Lys-75, His-96 and Asp-270 are potential residues of MtAlaDH involved in the catalytic activity. His-96 and Asp-270 are putative acid-base catalysts in the reaction while pyruvate is tightly bound to the side chains of two residues Arg-15 and Lys-75 [12,13]. Alanine dehydrogenase from alkaliphilic *Bacillus pseudofirmus* OF4 (OF4Ald) locates beside the alanine racemase gene and forms an operon for D-alanine metabolism [14]. A multiple protein sequence alignment of alanine dehydrogenases revealed that those four residues are highly conserved across different bacterial species. In addition, there is another conserved residue Lys-73 of OF4Ald that has attracted our attention as the mutation at this site played significant roles in catalytic reaction.

In order to unravel the biochemical properties of OF4Ald and a more particular knowledge of D-alanine synthesis and degradation in *B. pseudofirmus* OF4, single point mutation, protein expression, characterization and structural-based protein modeling of OF4Ald and its mutants were conducted. The functional properties of five conserved

* Corresponding authors.
E-mail addresses: jujiansong@126.com (J. Ju), Microbiology_hbnu@126.com (B. Zhao).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.enzmictec.2017.10.001
Received 9 May 2017; Received in revised form 25 September 2017; Accepted 10 October 2017

residues (Arg-15, Lys-73, Lys-75, His-96 and Asp-269) in the catalytic reaction, especially the residue Lys-73, were also explored in this study.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

E. coli DH5α and BL21(DE3) were used for DNA manipulation and recombinant protein expression, respectively. The expression plasmid pET-OF4Ald was constructed as previously described [15]. Pyrobest DNA Polymerase and all restriction enzymes were purchased from Takara (Takara, Japan). L-alanine and ß-NAD^+ (ß-nicotinamide adenine dinucleotide) were obtained from Roche (Switzerland). All other chemicals were of analytical grade.

2.2. DNA manipulations and alanine scanning mutagenesis

Based on sequence alignment results, five putative active site residues (Arg-15, Lys-73, Lys-75, His-96 and Asp-269) were chosen to be studied by alanine scanning mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). The primers used in this study are listed in Table 1. Plasmid pET-OF4Ald was used as a template for PCR amplification. The PCR products were digested with *Dpn* I and transformed into *E. coli* DH5α. The result of the site-directed mutagenesis was verified by DNA sequencing. The recombinant pET-OF4Ald and its five verified mutant plasmids were then transformed into *E. coli* BL21(DE3) for protein expression and purification.

2.3. Protein expression and purification

E. coli BL21 (DE3) harboring plasmids pET-OF4Ald, pET-R15A, pET-K73A, pET-K75A, pET-H96A and pET-D269A were cultured in 200 mL of fresh LB medium containing ampicillin (100 μg mL^{-1}) at 37 °C. When the optical density (OD_{600}) reached 0.6, isopropyl thio-β-D-galactoside (IPTG) was added at a final concentration of 1 mM, and the cell was cultivated at 30 °C for an additional 12 h.

Cells were collected by centrifugation at 6000*g* for 10 min at 4 °C. The protein was purified by Ni-NTA Superflow Cartridge (5 mL, Qiagen) according to manufacturer's instructions.

The elution was combined and loaded to the HiPrep 16/60 Sephacryl S-200 h column (1.6 × 60 cm; GE Healthcare) at 4 °C and eluted with 20 mM phosphate buffer (pH 8.0) containing 150 mM NaCl, 0.5 mM EDTA and 0.01% (v/v) 2-mercaptoethanol. Elution was performed at a flow rate of 0.5 mL min^{-1}. The protein fraction was pooled and dialyzed against the standard buffer (10 mM phosphate buffer pH 8.0, 0.5 mM EDTA and 0.01% 2-mercaptoethanol). Molecular mass of AldOF4 was determined by gel filtration chromatography as described previously [16]. Cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa) were used as molecular mass standard (Sigma, USA).

The purity of the purified protein was examined by SDS-PAGE. Protein concentration was determined using BCA Protein Assay Reagent Kit (Pierce, USA) with BSA as the standard.

Table 1
Primers used in this study.

Primer	Sequence(5'-3')	Description
Ald-F01	CACGCATATGATTATCGGTATTCCA	*ald* gene cloning
Ald-R01	AGCCTCGAGTGCTTGAACAGGTGTTTTC	
R15A-F01	AATGAAAACGCCGTAGCAAT	Arg-15→Ala-15
R15A-R01	ATTGCTACGGCGTTTTCATTA	
K73A-F01	ATGGTGATGGCAGTTAAAGA	Lys-73→Ala-73
K73A-R01	TCTTTAACTGCCATCACCAT	
K75A-F01	ATGAAAGTTGCAGAACCATTA	Lys-75→Ala-75
K75A-R01	TAATGGTTCTGCAACTTTCAT	
H96A-F01	ACATACCTTGCCCTAGCTGC	His-96→ Ala-96
H96A-R01	GCAGCTAGGGCAAGGTATGT	
D269A-F01	TAGCGATTGCCCAAGGCGGT	Asp-269→Ala-269
D269A-R01	ACCGCCTTGGGCAATCGCTA	
K73S-F01	ATGGTGATGTCAGTTAAAGA	Lys-73 → Ser-73
K73S-R01	TCTTTAACTGACATCACCAT	
K73E-F01	ATGGTGATGGAAGTTAAAGA	Lys-73 → Glu-73
K73E-R01	TCTTTAACTTCCATCACCAT	
K73Q-F01	ATGGTGATGCAAGTTAAAGA	Lys-73 → Gln-73
K73Q-R01	TCTTTAACTTGCATCACCAT	
K73R-F01	ATGGTGATGAGAGTTAAAGA	Lys-73 → Arg-73
K73R-R01	TCTTTAACTCTCATCACCAT	
K73Y-F01	ATGGTGATGTATGTTAAAGA	Lys-73 → Tyr-73
K73Y-R01	TCTTTAACATACATCACCAT	
K73F-F01	ATGGTGATGTTCGTTAAAGA	Lys-73 → Phe-73
K73F-R01	TCTTTAACGAACATCACCAT	

The underlined sequences are *Nde*I and *Xho*I restriction sites, respectively. Gray blackground letters are the mutated nucleotides.