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The research progress of alanine racemase

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Abstract: *Alanine Racemase (ALR) is a unique bacterial enzyme that racemises L-alanine to D-alanine in bacteria and plays a crucial role in the biosynthesis of bacterial peptidoglycan. In this paper, the structure, function and application of alanine racemase are described to provide a theoretical basis for the in-depth study of bacterial alanine metabolism and the development of related enzyme inhibitors.*

Keywords: *bacteria; alanine racemase; physiological function*

1. Introduction

L-alanine is an important amino acid involved in protein synthesis, dopamine synthesis and energy metabolism. It is also one of the essential amino acids for the human body and is vital for maintaining cell structure and function. D-alanine is one of the important components of the bacterial cell wall and is widely found in the bacterial cell wall, which is used to maintain bacterial structural stability and to protect the bacteria from external environmental stresses.[1]

Free D-alanine does not exist in nature, only free L-alanine. In prokaryotes, D-alanine is mainly derived from the conversion of L-alanine by alanine racemase. Alanine racemase is a pyridoxal-5'-phosphate-dependent bacterial enzyme that is involved in bacterial cell wall synthesis, providing it with the precursor D-alanine necessary for peptidoglycan cross-linking[2]. Since human cells do not have cell wall structures and Alr is widely found in bacteria[3]. Therefore, Alr becomes an ideal target for antimicrobial drug development.

2. Alanine racemisation enzymes in bacteria

Alanine Racemase (ALR, EC 5.1.1.1) is a PLP-dependent folding amino acid racemase that catalyses the conversion of L-alanine to D-alanine for the synthesis of peptidoglycan in the bacterial cell wall[4]. Since this enzyme is essential for bacteria, inhibition of the enzyme reduces the level of D-alanine in the bacterial body, thus blocking the synthesis of the bacterial cell wall and inhibiting bacterial growth[5]. and alanine racemase (Alr) is the sole source of D-alanine in many bacteria, and its inhibition is lethal[6]. This enzyme is widely distributed in prokaryotes and has been reported in strains of yeast (*Schizosaccharomyces pombe*) and thermophilic bacilli (*stearothermophilus*)[7], It is also present in some lower eukaryotes and plants, such as fungi, alfalfa (Medicago sativa L[8], but are not normally present in humans and other higher eukaryotes. Therefore, alanine racemase has become one of the ideal targets for the development of antimicrobial drugs that people are currently working on.

3. Genes encoding alanine racemase in bacteria

Since the first discovery of ALR in human Enterococcus faecalis in 1951, scientists have so far successively discovered the enzyme from a variety of bacteria[9], which produce D-Ala for PG biosynthesis.With the exception of Gly, all protein amino acids have at least one chiral alpha carbon and can therefore exist in two stereoisomeric forms: the left-handed (L) and right-handed (D) forms. Usually the L form is much more abundant than the D form, but only free L-amino acids exist in nature, not free D-alanine. In prokaryotes, D-alanine is converted from L-alanine mainly by the racemic conversion of L-alanine catalysed by alanine racemase (ALR)[10]. Bacteria contain one or two alanine racemase genes, Alr for constitutive expression and anabolism (biosynthetic D-Ala) and Dad X for induction and catabolism (catabolic D-Ala)[11]. The products synthesised by the former are involved in

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cell wall synthesis, while the latter are induced to be expressed by L-Ala. Usually Gram-negative bacteria encode both ALRs, such as Pseudomonas aeruginosa, Salmonella typhimurium and Escherichia coli, etc[12]. In contrast, most Gram-positive bacteria contain only a single Ala racemase gene[13]. To date, almost all bacteria contain the biosynthetic alanine racemase gene alr[14], which encodes an alanine racemase that is essential for the synthesis of D-alanine for peptidoglycan biosynthesis.

4. Structure of alanine racemase

Because of the low content and unstable nature of alanine racemase (ALR) in eukaryotes, the main focus is now on the study of ALR in prokaryotes, and the amino acid sequences of ALR from different bacterial sources are highly conserved, with up to 30%-50% homology[15]. The four-level structure of ALR proteins is divided into monomer, homodimer and trimer, which have been structurally purified and characterised from many bacteria, e.g., ALR proteins of Streptococcus thermophilus have a monomer structure. The ALR proteins of bacteria such as Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus pneumoniae are homodimers, consisting of two identical subunits, each of which is responsible for a catalytic site, and this dimeric structure helps to enhance catalytic activity and maintain stability. The ALR protein of B. subtilis is a trimer. Generally the catalytic activity of ALR is homodimer than monomer structure. In the case of Staphylococcus aureus[16]for example, its alanine racemase is a homodimer, consisting of two identical monomers interacting head to tail. Each Alr monomer has two distinct structural domains: an N-terminal α/β-folded barrel domain and a C-terminal structural domain consisting mainly of β-strands. The N-terminal structural domain pair consists of an eight-stranded α/β-barrel, at which the active site is located at the interface, and contains a pyridoxal 5′-phosphate (PLP)-binding residue covalently linked to Lys39.The PLP cofactor is linked to the α/β-barrel structural domain with a highly conserved catalytic lysine residues to form an internal aldehyde-diamine bond through which it covalently binds to the highly conserved catalytic lysine (Lys39) and extends towards the centre of the α/β-barrel. The C-terminal structural domain consists of residues 242-382 and has the secondary structure of the major β-strand. This region contains three antiparallel β-sheets, except for one β-sheet, where two of the five β-strands are parallel.

5. Physiological function of alanine racemase

5.1 Involved in the biosynthesis of bacterial cell wall peptidoglycan

Alanine racemase catalyses the conversion of L-Ala to D-Ala, a function that is essential for bacterial cell wall synthesis. Because D-Ala is an important component of peptidoglycan synthesis in the bacterial cell wall, it can be directly involved in the cross-linking of peptidoglycan in the form of D-Ala-D-Ala dipeptide. Therefore inhibition of alanine racemase activity reduces peptidoglycan biosynthesis, thereby affecting bacterial cell wall formation and preventing bacterial growth.Liu Dong[17]et al. found that Alr was essential for the growth of Aeromonas hydrophila, and that the absence of exogenous D-alanine in the culture medium led to growth arrest and cell wall damage in alr mutant strains, which in turn reduced their pathogenicity.Awasthy[18]et al. studied Mycobacterium tuberculosis and found that alr gene deletion mutant strains of Mycobacterium tuberculosis result in D-Ala deficiency and a rapid loss of viability, requiring the addition of exogenous D-Ala to the culture medium for growth. And the deficiency of this amino acid resulted in reduced survival of Mycobacterium tuberculosis in macrophages and mice.

5.2 Involved in spore development and germination

Alr is also involved in spore development and germination, and Alr proteins have been found in exospores of bacteria such as Clostridium difficile, Bacillus subtilis and Bacillus anthracis. Yasuda[19]et al. found that L-Ala induced germination of Bacillus subtilis spores, but the Alr-catalysed synthesis of D-Ala would compete with it to inhibit and thus render the spores dormant. In contrast, the Alr inhibitor D-cycloserine irreversibly inactivated Alr in bacteria, resulting in faster germination of L-Ala-induced spores than untreated spores[20], which suggests that Alr can contribute to the formation of spores and inhibit germination of Bacillus cereus by regulating the ratio of the two conformational alanines. Chesnokova[21]et al. showed that Alr converts L-Ala to D-Ala and thus prevents germination of Bacillus prespores. In the absence of Alr, germination of prespores occurs, resulting in the separation of cells from the parent, but they are not tolerant of mature spores. Therefore, D-Ala is considered to be

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an effective inhibitor of spore germination in this case. In conclusion, Alr by controlling spore germination can delay or prevent bacteria from entering an active growth state, thereby increasing their ability to survive under unfavourable conditions.

5.3 Regulation of bacterial biofilm formation

Bacterial biofilm is an adhesive layer produced by bacteria during the growth process and consists of bacteria that secrete Mucopolysaccharides, DNA and proteins and other substances secreted by bacteria. Its main function is to protect bacteria from the damage of the external environment and provide a stable growth environment. The formation of bacterial biofilm depends on bacterial adhesion, and alanine racemase is involved in regulating the alanine content on the bacterial surface, which in turn affects the bacterial adhesion ability. By regulating the activity or expression level of alanine racemase, the adhesion ability of bacterial biofilm can be regulated. Liu Shiyu[22]et al. found that the alr gene deletion strain of Streptococcus mutans had a sparser biofilm compared to the WT type. And with the increase of exogenous D-Ala concentration, it led to the increase of D-Ala content in the bacteria, which in turn led to the accumulation of D-Ala in the biofilm, thus enhancing the adhesion and agglomeration ability of the bacteria and making the biofilm denser.Hochbaum[23]et al. found that D-amino acids cause the release of proteins that are components of biofilms, thereby inhibiting biofilm breakdown. Alanine racemase catalyses the conversion of L-Ala to D-Ala exerting some inhibitory effect on biofilm catabolism.

5.4 Involved in regulating osmotic pressure in aquatic invertebrates

Aquatic invertebrates, such as sponges, crustaceans, and bivalves, typically live in aqueous environments and need to regulate osmotic pressure in their own bodies to adapt to the external environment. The enzyme alanine racemase is involved in the synthesis and metabolism of alanine in these animals[24], When environmental osmolality increases, alanine racemase catalyses the synthesis of alanine, increasing the intracellular concentration of alanine, which in turn raises the osmotic pressure, allowing the cell to retain water and avoid dehydration. Conversely, when ambient osmolality decreases, alanine racemase catalyses the degradation of alanine, decreasing the intracellular alanine concentration to avoid excessive water uptake. Overall, alanine racemase is involved in the synthesis and metabolism of alanine in aquatic invertebrates, thereby regulating the osmotic pressure inside and outside the cell to adapt to different water quality environments. This process plays a crucial role in maintaining the physiological functions and viability of these animals.

6. Determination of the enzymatic activity of alanine racemase

6.1 Determination of racemase activity based on changes in spin value absorption[25]

The higher the activity of the enzyme, the more pronounced the change of the spin value and absorbance. The system was: buffer, coenzyme PLP, different concentrations of substrate D- or L-alanine and bacteria, the above reaction solution was reacted and the supernatant was taken to determine the spin value. The spin values were determined at 365 nm using a Perkin-Elmer 241 rotameter. The enzyme reaction activity of the enzyme was estimated from the changes.

6.2 Determination of alanine racemase activity by the D-amino acid oxidation reaction method[26]

6.2.1 Racemic reaction

The racemic reaction system is: coenzyme PLP, different concentrations of substrate D- or L-alanine, add alanine racemase to start the reaction, then add 25 μL of 2 mol/L HCl to terminate the reaction, stand on ice for 2 min and then add the same volume of NaOH solution, to neutralise the excess acid, to obtain the racemic reaction product. Definition of enzyme activity: The amount of enzyme required to produce 1 μmol of substrate (L- or D-Ala) in 1 min is one unit of enzyme activity (U).

6.2.2 Oxidation reaction

The reaction system was 4-aminoantipyrine, D-amino acid oxidase, TOOS, Tris-HCl (PH=8.0), peroxidase, and appropriate amount of racemic reaction products. The reaction system was incubated at 37 ℃ for 20 min, and the absorbance value was measured at 550 nm with an enzyme meter.Definition

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of Alr enzyme activity: the amount of enzyme needed to catalyse the production of 1 μmol of D-alanine from L-Ala in 1 min was defined as one enzyme activity unit (U).

6.3 Determination of alanine racemase activity by enzyme kinetic parameters[27]

The alanine racemase activity was determined by measuring the D- and L-alanine content by HPLC. The racemisation reaction system contained: tris-HCl buffer, coenzyme PLP and different concentrations of substrate D- or L-alanine, and the reaction was started by adding alanine racemase. Borate buffer (PH=9.0) and N-tert-butyloxycarbonyl-L-cysteine as well as o-phthalaldeehyde were added to the reaction mixture, and 10 μl of the reaction mixture was injected into the HPLC Jasco after inducing fluorescence reaction for 2 min at 25°C, and D- and L-alanine eluted from the column were used as alanine-copper complexes. alanine were monitored as alanine-copper complexes at 254 nm. Enzyme activity was calculated by increasing the D- or L-alanine content.

7. Application of alanine racemase

7.1 Alanine racemase-as a target inhibitor

Alanine racemase is an important enzyme class with a wide range of applications in the biological sciences. It is capable of converting racemic alanine into isomeric natural alanine with efficient and highly selective catalytic properties. In recent years, studies have identified potential applications for alanine racemase - as target inhibitors[28]. Most of the identified alanine racemase inhibitors are suicide substrates that react with enzyme cofactors and tend to indiscriminately inhibit other PLP-containing enzymes, resulting in cytotoxicity, and are therefore not in clinical use. Yaping Wang[29]et al. screened ten novel non-substrate alanine racemase inhibitors by HTS. By kinetic studies, it was shown that homogallic acid is a competitive inhibitor of alanine racemase, while hydroquinone is a non-competitive inhibitor. Unlike DCS, these compounds do not inactivate other unrelated PLP-dependent enzymes, but achieve inhibition of Aspergillus hydrophilus by interacting with the alanine racemase active site. Lee[26]et al. reported that thiadiazolidinones can be 50% inhibitory concentrations of Mycobacterium tuberculosis alanine racemase (IC50) ranging from <0.03 to 28 μM.Ciustea[30]et al. also found that thiadiazolidinone inhibited MRSA growth by inhibiting alanine racemase activity, and its inhibition of alanine racemase activity at a 50% inhibitory concentration (IC50) ranged from 0.36 - 6.4 μM, and it irreversibly inhibited the enzyme.

7.2 Alanine racemase as a food-grade test indicator

Kumio[31]et al. use the alanine racemase gene as an indicator for the detection of Escherichia coli in food. This was done by amplifying a fragment of the alanine racemase gene containing the non-conserved sequence of the gene from E. coli genomic DNA by polymerase chain reaction, followed by digoxigenin labelling as a probe for the detection of E. coli. The food samples and bacteria were each treated in 0.1N NaOH containing 0.5% SDS for 10 minutes at 25°C and then spotted directly onto a nylon membrane for DNA hybridisation with a probe specific for E. coli. Various foods inoculated with E. coli K-12 showed positive signals, while no uninoculated foods showed any signals. Jingqi Chen[32]et al. use the alanine racemase-encoding gene as a selection marker for Bacillus subtilis. This was done by deleting the d-alanine racemase-encoding gene dal from the chromosome of Bacillus subtilis 1A751 using the Cre/lox system to produce a food-grade host. Subsequently, the plasmid-encoded selection marker dal was complemented in the food-grade host, resulting in successful expression of RDPE in the dal-deficient strain without the addition of d-alanine, and RDPE activity in the medium reached 46 U/ml at 72 h. Finally, the capacity of the food.

8. Alanine Racemase and Disease

8.1 Alanine racemase and anthrax

Bacillus anthracis is a gram-positive bacillus that can cause anthrax fever, a serious infectious disease. Bacillus anthracis is a spore-producing bacterium and these spores are highly durable and transmissible. Therefore, inhibiting the formation and survival of B. anthracis spores is essential for controlling the spread of anthrax and for prevention. McKevitt[33]et al. found that alanine racemase plays a role in the control of Bacillus anthracis sporulation enhancing the viability of Bacillus anthracis

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in spore formation and survival during interactions with mouse macrophages. The enzyme can influence spore formation and emergence by regulating the metabolic processes of alanine in B. anthracis cells. The conversion of L-alanine to D-alanine catalysed by alanine abrogating enzyme enhanced the survival of Bacillus anthracis during its interaction with mouse macrophages, suggesting its inhibitory effect on germination during interaction with these cells. Omotade[34]et al. found that the alanine racemase inhibitor DCS enhanced L-alanine-induced germination and effectively killed newly germinated spores. Thus, the induction of B. anthracis spore germination was enhanced by inhibiting alanine racemase activity, thereby preventing the survival of germinating spores.

8.2 Alanine racemase and tuberculosis

Tuberculosis is a chronic infectious disease caused by Mycobacterium tuberculosis. It mainly affects the lungs, but may also invade other organs such as the lymph nodes, kidneys, and bones. According to the World Health Organisation (WHO), Mycobacterium tuberculosis infects millions of people globally each year and causes millions of deaths[35]. D-cycloserine is a second-line anti-tuberculosis drug used in the treatment of tuberculosis, but it has serious toxic side effects[36]. Therefore, there is an urgent need to develop novel antimicrobial drugs against Mycobacterium tuberculosis. Alanine racemase, a phospho-5'-phosphate-dependent bacterial enzyme, provides bacteria with D-alanine, the precursor of peptidoglycan, which is involved in their cell wall synthesis. Therefore, alanine racemase was used as a target for the development of non-alanine analogue inhibitors, thereby minimising the off-target effect of D-cycloserine. Thiazolidinediones inhibit alanine racemase activity with IC50 concentrations below 1 μM. Nakatani[37]et al. used molecular modelling, in vitro MIC testing and direct measurements of enzyme activity to show that alanine racemase mutants of Mycobacterium tuberculosis confer DSC resistance. Thus, by investigating alanine racemase inhibitors, we expect to provide more options and innovations in TB treatment.

9. Conclusion

Bacterial cell wall biosynthesis is a target for future antimicrobial agent development, and several studies have shown that alanine racemase primarily catalyses the conversion of L-alanine to D-alanine, which is then involved in bacterial cell wall synthesis. In addition, there is much room for expansion of the application areas of alanine racemase. Future research will strengthen collaboration with other fields, such as materials science and food science, to create novel applications and further develop the scope of alanine racemase in the biomedical and chemical industries.

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