Biotechnology Advances 35 (2017) 657–668

Contents lists available at ScienceDirect

Biotechnology Advances

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

Research review paper

Breaking the mirror: L-Amino acid deaminase, a novel stereoselective biocatalyst

Gianluca Molla, Roberta Melis, Loredano Pollegioni*

Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, via J. H. Dunant 3, 21100 Varese, Italy
The Protein Factory, Centro Interuniversitario di Biotecnologie Proteiche, Politecnico di Milano and Università degli Studi dell'Insubria, Varese, Italy

ARTICLE INFO

Keywords:
Biocatalysis
Flavoproteins
Enantiomeric resolution
Structure-function relationships
Amino acids

ABSTRACT

Enantiomerically pure amino acids are of increasing interest for the fine chemical, agrochemicals and pharmaceutical industries. During past years L-amino acids have been produced from deracemization of DL-solution employing the stereoselective flavoenzyme D-amino acid oxidase. On the other hand, the isolation of corresponding D-isomer was hampered by the scarce availability of a suitable L-amino acid oxidase activity. On this side, L-amino acid deaminase (LAAD), only present in the *Proteus* bacteria, represents a suitable alternative. This FAD-containing enzyme catalyzes the deamination of L-amino acids to the corresponding α-keto acids and ammonia, with no hydrogen peroxide production (a potentially dangerous oxidizing species) since the electrons of the reduced cofactor are transferred to a membrane-bound cytochrome. Very recently the structure of LAAD has been solved: in addition to a FAD-binding domain and to a substrate-binding domain, it also possesses an N-terminal putative transmembrane α-helix (residues 8–27, not present in the crystallized protein variant) and a small α + β subdomain (50–67 amino acids long, named "insertion module") strictly interconnected to the substrate binding domain. Structural comparison showed that LAAD resembles the structure of several soluble amino acid oxidases, such as L-proline dehydrogenase, glycine oxidase or sarcosine oxidase, while only a limited structural similarity with D- or L-amino acid oxidase is apparent. In this review, we present an overview of the structural and biochemical properties of known LAADs and describe the advances that have been made in their biotechnological application also taking advantage from improved variants generated by protein engineering studies.

1. Introduction

Chiral pharmaceutical and agricultural compounds containing enantiomeric amine or amino acid groups can be produced using a variety of enzymes, such as amino acid dehydrogenases (EC 1.4.1.*X*), amine oxidases (EC 1.4.3.22), amino acid oxidases (EC 1.4.3.*X*), aminotransferases (EC 2.6.4.*X*), ammonia lyase (EC 4.3.1.*X*), and lipases (EC 3.1.1.*X*) (Turner, 2004; Pollegioni and Molla, 2011). Here, a chemoenzymatic cascade was used to deracemise a DL-mixture to the L-isomer by employing a D-amino acid oxidase (DAAO, EC 1.4.3.3) and a non-selective chemical reduction step to afford enantiopure L-amino acid after numerous cycles (Turner, 2004). In past years, the availability of recombinant wild-type and engineered variants of DAAO from different sources spurred its use in biocatalysis. Several groups have utilized evolved DAAO variants for different biotechnological applications, see (Khang et al., 2003; Sacchi et al., 2004; Caligiuri et al., 2006; Findrik and Vasić-Rački, 2007; Pollegioni et al., 2008; Ma et al., 2009; Wong et al., 2010). The most relevant industrial application of DAAO is the conversion of cephalosporin C into 7-aminocephalosporanic acid (6000 tons year^{-1}, a scaffold used to produce semi-synthetic cephalosporin antibiotics) (Henderson et al., 2008; Pollegioni et al., 2008; Pollegioni et al., 2013a, b). DAAO is also used in biosensors, in the resolution of natural (such as methionine and phenylalanine) and synthetic (such as naphthylalanine) amino acid racemic solutions (Caligiuri et al., 2006; Findrik and Vasić-Rački, 2007; Martínez-Rodríguez et al., 2010), and in keto acids production (Song et al., 2016).

Theoretically, the same approach can be used to produce D-amino acids simply by using an amino acid oxidase with reverse stereoselectivity. L-Amino acid oxidases (LAAO, EC 1.4.3.2) are well known

Abbreviations: α-KG, 2-ketoglutaric acid; CTAB, cetyltrimethylammonium bromide; DAAO, D-amino acid oxidase; ΔN-LAAD, N-terminal LAAD deletion variant; FBD, flavin binding domain; LAAD, L-amino acid deaminase; LAAO, L-amino acid oxidase; PAL, phenylalanine ammonia lyase; PmaLAAD, *Proteus myxofaciens* type-I LAAD; PmirLAAD, *Proteus mirabilis* type-I LAAD; Pm1LAAD, *Proteus mirabilis* type-II LAAD; PMS, phenazine methosulfate; PPA, phenylpyruvic acid; pvLAAD, *Proteus vulgaris* type-II LAAD; SBD, substrate binding domain

* Corresponding author.
E-mail address: loredano.pollegioni@uninsubria.it (L. Pollegioni).

http://dx.doi.org/10.1016/j.biotechadv.2017.07.011
Received 10 March 2017; Received in revised form 4 July 2017; Accepted 30 July 2017
Available online 03 August 2017

FAD-containing flavooxidases which catalyze the stereoselective oxidative deamination of L-amino acids into the corresponding α-keto acids and ammonia; the reduced flavin is reoxidized by O_2, generating H_2O_2 (Pollegioni et al., 2013a, b). The reaction and the mechanism underlying substrate dehydrogenation by LAAO are identical to those of D-amino acid oxidase (Umhau et al., 2000; Pawelek et al., 2000). LAAO catalyzes the dehydrogenation of the L-amino acid through a hydride transfer mechanism by steering the trajectory of the αC–H orbital of the substrate to the LUMO of the FAD cofactor N(5), without direct involvement of active site residues (Pawelek et al., 2000). LAAO activity has been identified in mammals, snakes, fishes, insects, molluscs, algae, fungi, and bacteria (Lukasheva et al., 2011; Yu and Qiao, 2012; Pollegioni et al., 2013a, b). Snake venom LAAOs are the best-known members of this family (Du and Clemetson, 2002). Unfortunately, LAAOs proved to be inappropriate for biocatalysis owing to the difficulties associated with overproduction of snake venom LAAOs in recombinant hosts (which also prevented engineering studies) and the limited substrate acceptance of the microbial counterparts (Pollegioni et al., 2013a, b). Actually, different LAAOs are known to have narrow substrate acceptance, *e.g.*, L-aspartate oxidases (EC 1.4.3.16), L-glutamate oxidase, L-lysine oxidase, L-phenylalanine oxidase, and L-tryptophan oxidase.

L-Amino acid deaminase (LAAD) represents a suitable alternative to the functionally similar LAAOs. This enzyme employs FAD to catalyze the deamination of L-amino acids, yielding the corresponding α-keto acids and ammonia: the electrons of the reduced cofactor are transferred to a cytochrome with no hydrogen peroxide production (see Scheme 1).

LAADs can be used as the key element to develop the following interesting biotechnological applications:

a) novel biocatalytic processes. A main application of LAAD is the production of optically pure D-amino acids by resolving DL-racemic mixtures, as well as the production of the corresponding α-keto acids;
b) biosensors. DAAO and LAAO activity has been applied as a biological tool for analytically determining the level of amino acid enantiomers by using colorimetric or amperometric biosensors (Domınguez et al., 2001; Inaba et al., 2003; Stefan et al., 2003; Rosini et al., 2008; Pernot et al., 2008; Pollegioni and Molla, 2011; Pollegioni et al., 2013a, b). LAAD variants could replace LAAO in this application because of their substrate acceptance plasticity, although detection methods based on H_2O_2 production could not be used;
c) diagnostics and therapeutics. LAAD could replace LAAOs in different applications related to human health. L-Glutamate oxidase was used to assay alanine and aspartate aminotransferase activity in biological samples by applying a photometric method (Sukhacheva and Zhuravleva, 2004); L-lysine oxidase was reported to suppress DNA, RNA, and protein synthesis in various tumor cell lines *in vitro* and to affect cell cycle progression (Lukasheva and Berezov, 2002); and LAAO was demonstrated to increase the antitumor activity of melphalan by depleting large neutral amino acids in murine plasma (Moynihan et al., 1997) and to possess antibacterial and antiviral activity.

Although the reaction catalyzed by LAAD is of both scientific and practical interest, the structural-functional relationships in this enzyme (and its biological significance) have only recently been investigated. In this review, we present an overview of the structural and biochemical properties of LAAD and describe the advances that have been made in biotechnological application.

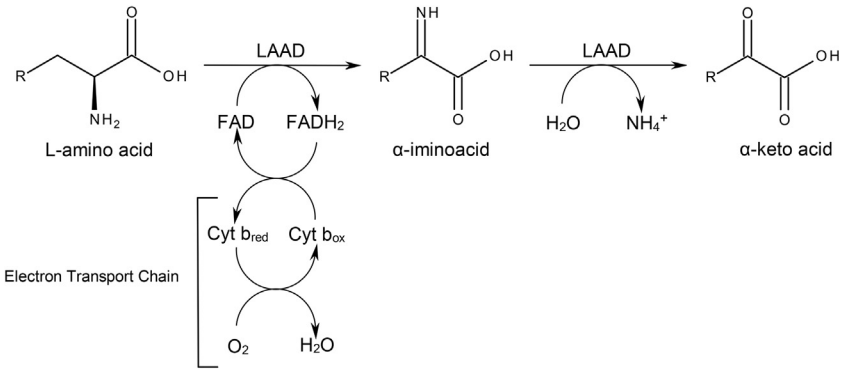
2. Structure-function relationships in LAAD

Only bacteria belonging to the genus *Proteus* express the LAAD enzyme. Each *Proteus* species expresses two different types of this membrane-associated enzyme, which share a significant sequence identity (~57%) but differ in substrate specificity. Type-I LAADs preferentially oxidize bulky aliphatic and aromatic amino acids, while type-II LAADs have significant activity toward charged (mainly basic) amino acids, such as histidine and arginine (Takahashi et al., 1999; Baek et al., 2011). Phylogenetic analysis demonstrates that proteins with a significant homology to *P. myxofaciens* LAAD (> 28% of sequence identity) are only present in the α-, β-, and γ-subphyla of proteobacteria. The products of these genes are classified as putative flavoprotein oxidases or deaminases with unknown function. Interestingly, the two *Proteus* type-I or type-II LAADs share only a very low overall degree of sequence identity with canonical L- or D-amino acid oxidases (between 13.9 and 16.4%, respectively) (Motta et al., 2016).

Whereas BLAST analysis has shown the presence of genes coding for putative type-I or type-II LAADs in the genome of almost all *Proteus* species, up to now only four different LAADs have been expressed in a recombinant form and characterized from a biochemical point of view: the type-I proteins from *P. mirabilis* (PmirLAAD) and *P. myxofaciens* (PmaLAAD) and the type-II enzymes from *P. vulgaris* (PvLAAD) and *P. mirabilis* (Pm1LAAD) (Massad et al., 1995; Baek et al., 2011; Motta et al., 2016; Ju et al., 2016).

2.1. Substrate specificity and electron acceptors

From a functional point of view, one main difference between type-I and type-II LAADs concerns substrate preferences. Type-I LAADs (*e.g.*, PmaLAAD) show the highest catalytic efficiency on bulky and hydrophobic substrates, such as L-Phe (the k_{cat} for this substrate is 8.3 s^{-1} with a K_m of 3.27 mM), L-Leu, L-Met, and L-Trp. PmaLAAD also shows a significant rate of activity on the polar amino acid L-Cys (43% of the activity on L-Phe) and a low rate (< 10%) on small apolar or charged amino acids (Motta et al., 2016). PmirLAAD shows an overall lower kinetic efficiency with a k_{cat} of 1.4 s^{-1} and a K_m of 26.2 mM on L-Phe (Table 1). Notably, these values could be affected by the purification protocol employed, see below (Hou et al., 2015).



Scheme 1. Reaction catalyzed by LAAD on L-amino acids. The reduced flavin transfers the electrons on a membrane-bound acceptor, such as a cytochrome *b*-like protein, with no hydrogen peroxide production.