Bacterial d-amino acid oxidases: Recent findings and future perspectives

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D-aminocid acid oxidase (DAO) is a flavin enzyme that catalyzes the oxidative deamination of d-aminocid acids. This enzyme has been studied extensively both biochemically and structurally as a model for the oxidase-dehydrogenase class of flavoproteins. This enzyme also has various applications, such as the determination of d-aminocid acids and production of building blocks for a number of pharmaceuticals. DAO has been found mainly in eukaryotic organisms and has been suggested to play a significant role in various cellular processes, one of which includes neurotransmission in the human brain. In contrast, this enzyme has not been identified in prokaryotic organisms. Some studies have recently identified and characterized DAO enzyme in some actinobacteria. In addition, a genome database search reveals a wide distribution of DAO homologous genes in this bacterial group. The bacterial DAOs characterized so far have certain distinct properties in comparison to eukaryotic DAOs. These enzymes also exhibit some important applicable properties, suggesting that bacteria could be used as a source for obtaining novel and useful DAOs. The physiological function of bacterial DAO have been proposed to include the degradation of non-canonical d-aminocid acids released from cell wall, but is still largely unknown and need to be studied in depth.

Introduction

DAO (EC 1.4.3.3) catalyzes the oxidative deamination of neutral and basic d-aminocid acids and contains a non-covalently bound flavin adenine dinucleotide (FAD) moiety as a cofactor. This enzyme reaction produces imino acids from d-aminocid acids concomitant with the reduction of FAD (Fig. 1). The imino acids are then non-enzymatically hydrolyzed to give corresponding α-keto acids and ammonium. The reduced FAD is reoxidized by molecular oxygen to produce hydrogen peroxide. The substrate stereoselectivity of DAO is specific, and thus l-aminocid acids are not accepted as substrates. In addition, this enzyme exhibits a negligible or no activity toward acidic d-aminocid acids, which are substrates for another flavin oxidase d-aspartate oxidase (DDO, EC 1.4.3.1). DAO has been extensively studied for its biochemical and structural properties as a model enzyme of the oxidase-dehydrogenase class of flavoproteins. Further, the enzyme can be utilized for a broad range of applications, such as the determination of d-aminocid acids, production of building blocks for pharmaceuticals, and diagnosis and treatment of certain diseases, now desiring a highly stable DAO.

DAO was first identified by Dr. Krebs in pig kidney, and since then this enzyme has also been found in various eukaryotic organisms, such as fungi, nematodes, fish, plants, animals, and human. The homologs of this enzyme show amino acid sequence variations; however, some of the residues responsible for substrate binding and interactions with FAD are highly conserved. The eukaryotic DAOs possess a peroxisome-targeting signal peptide at their carboxy terminus to localize in the peroxisome for removing the hydrogen peroxide produced by the enzyme reaction. Moreover, DAO plays an important role in various cellular processes in the eukaryotic organisms. In fungi, the enzyme degrades d-aminocid acids to utilize them for cell growth and to protect them from the toxic effect of d-aminocid acids. In animals, this enzyme plays a role in the
A homology search in a prokaryotic genome database reveals that DAO homologous genes are widely distributed in various bacteria, except for Archaea that appear to have no homologous genes. The bacterial species having DAO homologous genes are predominantly members of the phylum *Actinobacteria*, which include *Arthrobacter protophormiae* and some pathogenic bacteria, such as *Mycobacterium* spp. and *Nocardia* spp. 

In this addendum, we have described the occurrence, structure, and enzymatic properties of bacterial DAOs. We also discuss the putative physiological role and the potential biotechnological applications of the bacterial DAOs.

### Occurrence of Bacterial DAOs and their Homologous Genes

The first bacterial DAO was reported by Gueueke et al. 

They isolated some bacterial species from soil samples that exhibited DAO activity. Among these species, the bacterium that exhibited the highest DAO activity was identified as *Arthrobacter protophormiae* based on its 16S rRNA gene sequence. The crude extract from the bacterial cells however exhibits only a small activity (3.0 mU/mg-protein) even against the most preferred substrate i.e. d-methionine. In contrast, DAO activities in yeast are detected at approximately 1.0 U/mg-protein. 

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### Structure of Bacterial DAOs

The primary sequence of bacterial DAO was first identified in *A. protophormiae* DAO (ApDAO) by cloning the encoding gene. 

The primary structures of ScDAO and RxDAO were identified from a genome database, but the sequence of RxDAO is slightly different from that of the isolated gene. 

The primary sequence of these bacterial DAOs show high amino acid identities, approximately 38–46%. However, these percentage identity values are only slightly higher in comparison to those obtained for eukaryotic DAOs (approximately 30–36%). In the primary structure of the DAOs, the functional amino acid residues are conserved between eukaryotic and bacterial DAOs. These include the dinucleotide-binding motif (GXGXXG) at the N-terminal region and an arginine, a tyrosine, and a glycine residues binding to the α-carboxy and α-amino groups of the substrate.

However, peroxisome-targeting signal peptides found in eukaryotes at the C-terminus is lacking in the bacterial enzyme, resulting in a shorter length of the amino acid sequence (approximately a difference of 20–40 amino acid residues).

ApDAO and RxDAO are dimeric and monomeric proteins, respectively. 

Almost all eukaryotic DAOs are dimers, except the enzymes found in rat and yeast, *Candida boidinii*, are monomers. 

Animal DAOs are arranged as head-to-head dimers and the yeast *Rhodotorula gracilis* DAO (RgDAO) is a head-to-tail dimer. The head-to-tail dimer formation in RgDAO results due to an extra stretch of amino acids not found in other eukaryotic DAOs. This additional stretch is also not found in ApDAO, suggesting its head-to-tail dimer formation. It has been proposed that the surface potential of the dimer interface might be involved in the interactions between the monomers, i.e., the positively and negatively charged interfaces possibly cause a weak and a strong interaction, respectively.

This argument is further strengthened by the fact that the surface of RxDAO model structure is abundant in positively charged residues.

The crystal structures of some eukaryotic DAOs have been reported, whereas no crystal structure for bacterial DAOs have been determined. However, a 3-dimensional structure model for RxDAO has been built. In this model, the substrate binding at the active site is very similar to the one in eukaryotic DAOs. The α-carboxy and α-amino groups of the substrate interact with Arg272 and Tyr217 residues, and with the carbonyl oxygen of Gly299, respectively (Fig. 2).

In addition, similar to eukaryotic DAOs, the side chain of the substrate is covered by the hydrophobic amino acid residues of the enzyme. However, the composition of these hydrophobic residues is different from eukaryotic ones, thus resulting in a different active site structure that might provide different substrate specificity.
parameters of bacterial DAOs have been compared to that of animal DAOs. The kinetic parameters for ApDAO, only $K_m$ value (1 mM for d-methionine) has been reported. This value is comparable to that of eukaryotic DAOs. $^{10}$

Enzymatic Properties of Bacterial DAOs

The enzymatic properties of bacterial DAOs are summarized in Table 1. Three bacterial DAOs analyzed so far are divided into 2 groups based on substrate specificity. ScDAO and RxDAO show higher activities toward branched chain d-amino acids and d-methionine. $^{10,12}$ In contrast, ApDAO exhibits higher activities for basic d-amino acids as well as d-methionine. $^{11}$

The highest activity shown by ApDAO is for the most preferred substrate for this enzyme. The $K_m$ value is one order magnitude lower in comparison to that for preferred substrates of other DAOs. Among the kinetic parameters for ApDAO, only $K_m$ value has been reported. This value is comparable to that of other eukaryotic DAOs.

Although, the optimum temperature for ApDAO has been not reported, the incubation of the enzyme at 50°C for 2 min decreases the activity by 50%. $^{11}$

The highest activity shown by ApDAO is approximately 180 U/mg with d-methionine, which is comparable to the activity of yeast DAOs. In contrast, RxDAO exhibits an activity of approximately 20 U/mg with d-valine, which is similar to that of animal DAOs. The kinetic parameters of bacterial DAOs have been investigated in detail only for RxDAO. The highest calculated $k_{cat}$ value (53 s$^{-1}$) for RxDAO is obtained for d-tyrosine as substrate, but the apparent $k_{cat}$ value may be much lower, as d-tyrosine exhibits substrate inhibition. $^{10}$ Similar substrate inhibition was also found in human DAOs. $^{19}$

The specific activity of the crude extract is 0.063 U/mg-protein. Further, RxDAO is also produced mainly in the inclusion bodies, and the soluble enzyme occupies approximately 2% of the total soluble proteins with 7.05 U/g-wet cells and 33.7 U/L-culture medium under optimized conditions. $^{12}$ The specific activity of the crude extract is 0.095 U/mg-protein. Further, RxDAO is also produced mainly in the inclusion bodies, and the soluble enzyme occupies approximately 2% of the total soluble protein. $^{10}$ The decrease in the expression temperature increases the soluble enzyme content to approximately 6%; however, this results in a simultaneous decrease in the activity. The incubation of the crude extract at 70°C increases the activity, implying the presence of a folding intermediate. The specific activity of RxDAO in the crude extract (0.063 U/mg-protein) is comparable to that of ScDAO but is considerably lower in comparison to that of ApDAO. The activities for RxDAO per culture and per wet cell weight are 32.3 U/L and 3.96 U/g, respectively.
**Possible roles of Bacterial DAOs**

Bacteria produce d-amino acids, usually d-alanine and d-glutamate, to synthesize the peptidoglycans in the cell wall. Recently, the other d-amino acids, such as d-valine, d-leucine, d-tyrosine, d-methionine, and d-tryptophan are found to be produced by various bacterial species and are called non-canonical d-amino acids. These non-canonical d-amino acids have been shown to be incorporated into the bacterial cell wall and have been suggested to be involved in cell wall remodeling and biofilm disassembly.

ScDAO preferentially catalyzes some of the non-canonical d-amino acids as substrates, giving an indication that this enzyme was possibly involved in cell wall remodeling or biofilm disassembly through the degradation of the d-amino acids. The addition of these d-amino acids in the culture medium of *S. coelicolor* retards the cell growth and alters the cell shape, suggesting the inhibition of cell wall synthesis. However, these non-canonical d-amino acids do not affect the biofilm formation (attachment to solid surfaces in the standing liquid cultures). In this bacterium, several putative peptidase genes possibly involved in the cell wall degradation are located around the DAO gene. These findings suggest the possibility that ScDAO may play an important role in the degradation of non-canonical d-amino acids released from the cell wall. Although the role of other bacterial DAOs has yet to be analyzed, the largely different activity and substrate specificity of ApDAO suggests that ApDAO might have a distinct physiological role in comparison to ScDAO.

**Potential Applications of Bacterial DAOs**

DAO is an effective catalyst that can be utilized in diverse applications. The potential applications of DAO are as follows: the determination of d-amino acids, the optical resolution of amino acid racemate, the analysis of optical purity of l-amino acids, the production of α-keto acids, the production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin C, the conversion of d-amino acids to l-amino acids, the production of unnatural amino acids, the diagnosis of psychotic disorders, and the treatment of cancer. Of these, an important commercialized application of DAO is the production of 7-ACA, which is an important intermediate in the production of semisynthetic cephalosporins that are one of the best-selling antibiotics. However, ApDAO is unable to catalyze cephalosporin C, and it is unknown with clarity whether ScDAO or RxDAO could catalyze the compound. The determination of d-amino acids in various samples has recently gained much attention, because d-amino acids are found to play a significant role in various biological processes, and their presence in different foods and beverages can affect the taste and nutritional value of these items. Currently, d-amino acids are mainly determined using high-performance liquid chromatography; while, the enzymatic determination method using DAO has also been developed for the purpose of more rapid and convenient estimation. However, each d-amino acid cannot be determined individually using eukaryotic enzymes because of their broad substrate specificities. In contrast, the substrate specificity of bacterial DAOs is relatively narrow. If protein engineering deletes only the activity of ApDAO toward d-methionine, the engineered ApDAO enzyme could be useful for determining the basic d-amino acids. RxDAO has a higher binding affinity toward branched chain d-amino acids, which might be useful for determining lower concentrations of the d-amino acids in a sample. In addition, the higher stability of RxDAO may be useful as a scaffold for creating a novel DAO suitable for each application mentioned above.

**Conclusions and Future Perspectives**

DAO is a versatile enzyme that not only acts as a model enzyme for the flavin-dehydrogenase class of flavoproteins but also acts as a catalyst in various applications. This enzyme was found in animal tissues approximately 80 y ago, and it was until recently identified only in eukaryotic organisms. Therefore, the studies and the applications of DAO have been conducted in eukaryotes. However, recent studies reveal the presence of DAOs in bacteria and suggest the presence of this enzyme in a wide variety of bacterial species. Bacterial DAOs have been shown to possess unique enzymatic properties, such as, substrate specificity, substrate-binding affinity, and stability, in comparison to eukaryotic enzymes, indicating their potential use in various applications. On the other hand, the physiological role of bacterial DAOs is still largely unknown, as various studies on these enzymes have commenced only recently. Further studies...
could help to understand the physiological functions of not only these enzymes but also the non-canonical d-amino acids in bacteria. In addition, several other key topics might be considered in future research. One of them is to understand the mechanism responsible for the higher thermal stability of RxDAO. This study might help to improve the stability of valuable DAOs, especially used for the production of the antibiotic intermediate. It is also important to investigate the mechanism of the higher activity of ApDAO toward basic d-amino acids. This investigation might contribute to understand the substrate recognition mechanism of DAO and to the creation of biosensor for the enzymatic detection of basic d-amino acids. To elucidate these mechanisms, the determination of crystal structure of these bacterial DAOs would be a critical challenge. Furthermore, because it has been shown that d-amino acids or their derivatives are possibly used to prevent the infection by pathogenic bacteria, it is also important to investigate DAO as well as d-amino acids metabolizing enzymes of pathogenic bacteria, such as Nocardiopsis spp. and Mycobacterium spp. These future researches on bacterial DAOs would not only provide us new fundamental findings of the enzyme but also contribute to expand the use of this enzyme in a wide variety of applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

Funding

A part of our works in this article was supported by a Grant-in-Aid for Scientific Research (C) (23580106), provided to ST from the Japan Society for the Promotion of Science.

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