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Biochimica et Biophysica Acta 1764 (2006) 1577–1585
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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A novel type of lysine oxidase: L-lysine-ε-oxidase

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Received 20 April 2006; received in revised form 16 August 2006; accepted 16 August 2006
Available online 3 September 2006

Abstract

The melanogenic marine bacterium *M. mediterranea* synthesizes marinocine, a protein with antibacterial activity. We cloned the gene coding for this protein and named it *lodA* [P. Lucas–Elío, P. Hernández, A. Sanchez-Amat, F. Solano, Purification and partial characterization of marinocine, a new broad-spectrum antibacterial protein produced by *Marinomonas mediterranea*. Biochim. Biophys. Acta 1721 (2005) 193–203; P. Lucas-Elío, D. Gómez, F. Solano, A. Sanchez-Amat, The antimicrobial activity of marinocine, synthesized by *M. mediterranea,* is due to the hydrogen peroxide generated by its lysine oxidase activity. J. Bacteriol. 188 (2006) 2493–2501]. Now, we show that this protein is a new type of lysine oxidase which catalyzes the oxidative deamination of free L-lysine into 6-semialdehyde 2-aminoadipic acid, ammonia and hydrogen peroxide. This new enzyme is compared to other enzymes related to lysine transformation. Two different groups have been used for comparison. Enzymes in the first group lead to 2-aminoadipic acid as a final product. The second one would be enzymes catalyzing the oxidative deamination of lysine releasing H_2O_2, namely lysine-α-oxidase (LαO) and lysyl oxidase (Lox). Kinetic properties, substrate specificity and inhibition pattern show clear differences with all above mentioned lysine-related enzymes. Thus, we propose to rename this enzyme lysine-ε-oxidase (*lod* for the gene) instead of marinocine. Lod shows high stereospecificity for free L-lysine, it is inhibited by substrate analogues, such as cadaverine and 6-aminocaproic acid, and also by β-aminopropionitrile, suggesting the existence of a tyrosine-derived quinone cofactor at its active site.

Keywords: L-lysine; Amino acid oxidases; Deamination; Antibacterial activity; Hydrogen peroxide

1. Introduction

L-amino acid oxidases (LAOs, E.C. 1.4.3.2) are a family of flavoproteins widely occurring in nature that catalyze oxidative deamination of L-amino acids to produce the corresponding α-keto acids, hydrogen peroxide and ammonia [1] (Eq. (1)). Although their structure, substrate specificities and functions show important variations, one of their main common properties is the cytotoxic and bactericidal action due to hydrogen peroxide formation [2,3].

$$\text{R-CH}(NH_2)\text{-COOH} + H_2O + O_2 \rightarrow \text{R-CO-COOH} + H_2O_2 + NH_3 \quad (1)$$

Most of LAOs prefer as substrate hydrophobic and neutral amino acids, such as leucine or methionine, and they show very low affinity for basic amino acids. However, an oxidase specific for L-lysine called L-lysine-α-oxidase, (E.C. 1.4.3.14, LαO) was isolated from the fungus *Trichoderma viride* [4]. This oxidase has been later described as a promising antibacterial, cytotoxic and antitumor agent [5]. In addition to LαO, another LAO with protective functions named escapin that shows preference for the basic amino acids L-lysine and L-arginine has been recently described in sea hare [6].

In relation to lysine oxidases producing hydrogen peroxide, lysyl oxidase (also named protein-lysine-6-oxidase, EC 1.4.3.13, Lox) is a different enzyme from LAO and LαO. This enzyme

Abbreviations: AAN; aminoacetonitrile; βAPN; β-aminopropionitrile; LAO; L-amino acid oxidase; LαO; Lysine-α-oxidase; Lat; Lysine-ε-amino-transferase; Lod; Marinocine (Lysine-ε-oxidase); Lox; Lysyl oxidase; LTQ; Lysyl-Tyrosyl-Quinone; SDH; saccharopine dehydrogenase; SSAO; semicarbazide sensitive amino oxidase; TDQC; tyrosine-derived quinone cofactors; TLC; Thin layer chromatography

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doi:10.1016/j.bbapap.2006.08.014

catalyzes the ε-oxidative deamination of lysyl residues in mammalian sclerotic proteins (Eq. 2), especially collagen and elastin, to yield allysyl residues that rapidly cross-link those proteins during the formation of the extracellular matrix. These reactions play an important role in the development, elasticity and extensibility of the connective tissue.

$$\text{Protein-}(CH_2)_3\text{-}CH_2\text{-}NH_2 + O_2 \rightarrow \text{Protein-}(CH_2)_3\text{-CHO} + H_2O_2 + NH_3 \quad (2)$$

Associated with the release of hydrogen peroxide, Lox was early described having a tumor suppressor activity [7]. More recently, an opposite role in cancer has been found, since Lox seems to be required for hypoxia-induced metastasis [8] and it has been now considered a target for treatment of aggressive neoplastic growth [9].

However, Lox is not only expressed in mammalian tissues, since the yeast *Pichia pastoris* also contains an oxidase with much more affinity for endopeptidyl lysine residues and diamines than for the free basic amino acids, L-lys or L-orn [10,11]. Lox does not depend on flavin, and it is frequently considered an unusual amine oxidase rather than a LAO because of its molecular properties and substrate specificity. Thus, amine oxidases (EC 1.4.3.6) and Lox are copper-enzymes and generally contain a tyrosine-derived quinone as cofactor [12]. In addition, Lox is also active not only on lysyl-containing peptides but also on free amines, such as cadaverine or benzylamine [13,14]. Thus, Lox is currently classified within the SSAO (semicarbazide sensitive amino oxidase) family [15,16].

We recently reported the existence in the melanogenic marine bacterium *M. mediterranea* of a protein with antibacterial activity that we named marinocine [17]. A similar protein is synthesized by another marine bacterium, *Pseudoalteromonas tunicata* [18]. Further characterization of marinocine showed that its antibacterial activity is mediated by hydrogen peroxide, and it was only active in L-lysine-containing media. Bearing in mind this catalytic activity, we named the cloned gene coding for it as *lodA* for lysine oxidase [19].

In this study we have characterized the enzymatic activity of marinocine in comparison with the previously described amino acid oxidases and other enzymes involved in lysine metabolism. We have found that marinocine is a new type of lysine oxidase with specific properties and clear differences to those enzymes. Marinocine catalyzes the direct one-step oxidative deamination of free L-lysine into 6-semialdehyde-2-aminoadipic acid. Inhibition and spectral data suggest that it can be classified in the group of the TDQC-containing amino acid oxidases. We propose to name this enzyme L-lysine-ε-oxidase (or L-lysine-6-oxidase).

2. Materials and methods

2.1. Obtention and purification of marinocine

Marinocine was obtained from supernatants of *Marinomonas mediterranea* cultures growth in marine minimal medium as previously described [17]. After 48 h, 30 ml of the culture were centrifuged at 4000×*g* for 30 min and the cell pellet was discarded. 60 ml of 96% ethanol were added to the supernatant and the ethanolic suspension was centrifuged at 19000×*g* at 4 °C for 20 min. The protein pellet obtained was dried and resuspended in 2 ml of 0.1 M sodium phosphate buffer, pH 7. That concentrate preparation was submitted to DEAE-Sephadex A-50 chromatography and eluted with a NaCl gradient. Semipurified marinocine eluted as a sharp peak at 0.9 M NaCl. The active fractions obtained were pooled and concentrated using 50-kDa centrifugal filter units (Ultrafree, Millipore) before being submitted to gel permeation chromatography on Sephacryl S-200 HR equilibrated in 0.1 M phosphate buffer pH 7. SDS-PAGE analysis and Coomassie Blue stain of the active fractions showed a purified protein of apparent mass around 140 kDa. The purification factor was 56 in relation to the specific activity in the supernatant of the cultures, and the yield of the purification process was 35%, in good agreement with former purifications of marinocine [17].

2.2. Antibiograms

A suspension of *E. coli* DH5α (OD_{600}=0.2) was seeded on Mueller Hinton plates supplemented with 1% NaCl. 20 µl of marinocine samples were loaded into 6-mm discs of Filter Paper Backing (BioRad) and allowed to air-dry before placing them onto the agar plate. Plates were then incubated for 48 h at 25 °C and the diameter of the inhibition ring was measured. One unit of marinocine gives an inhibitory ring of 8.06 mm. All details about the antibiogram assay, and correlation between growth inhibition diameter (mm) and marinocine units have been previously described [17].

2.3. Enzymatic assays

2.3.1. Fluorimetric determination of H_2O_2 production

We have used 3 different assays to estimate the lysine oxidase activity and to characterize the reaction products. All assays were performed in duplicate with good reproducibility. The fluorimetric assay was the most sensitive one. This method was first reported for determination of Lox activity using 1,5-diaminopentane as substrate. It is based on the determination of the H_2O_2 by detection of oxidized Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) using a horseradish peroxidase-coupled reaction [14]. In our conditions, we used 50 µM L-lysine as routine substrate, 0.05 mM Amplex red (Molecular Probes, A-22188) and 0.1 U/ml of peroxidase in the reaction mixture. For affinity and inhibition studies, variable concentrations of L-lys or putative alternative substrates were also used. Reactions were carried out for 15 min in 96 wells ELISA plates in 100 µl of total volume per assay. Amplex red oxidation was followed using an excitation filter of 550 nm and emission filter at 590 nm. Background fluorescence due to the slow spontaneous oxidation in the absence of L-lysine was subtracted. Fluorimetric units are defined very differently to antibiogram units, but a comparison between both has been formerly published [19].

2.3.2. α-Keto acid determination

This method consisted in the formation of the semicarbazone derivative obtained by the reaction of 0.5 M semicarbazide with the keto-acid obtained after the lysine oxidative deamination [20]. LαO yields 2-keto-6-aminocaproic acid and its semicarbazone is estimated by absorbance increase at 248 nm ($\varepsilon = 10160 \pm 240\ M^{-1}\ cm^{-1}$). The routine reaction mixture was 0.8 ml containing 1 mM L-Lys and 0.5 M semicarbazide in 0.1 M sodium phosphate buffer pH 7.4. To facilitate comparison, LαO units are expressed as commercially defined enzymatic IU (1 unit is the amount of enzyme producing 1 µmol of product/min at pH 8).

2.3.3. Aldehyde determination

This method allows the determination of L-lysine oxidation in the ε-amine group. It consisted in the quantitation of the 6-semialdehyde 2-aminoadipic acid by coupling the reaction with aldehyde dehydrogenase and NAD^+ to detect the NADH appearance at 340 nm [21]. The routine reaction mixture was 0.8 ml containing 1 mM L-Lys, 0.5 mM NAD^+, 0.5 mM β-mercaptoethanol and 0.05 mU of yeast aldehyde dehydrogenase (E.C. 1.2.99.3, Sigma) in 0.1 M sodium phosphate buffer pH 7.5.