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Biochimica et Biophysica Acta 1764 (2006) 1577–1585
BIOCHIMICA ET BIOPHYSICA ACTA
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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.

3. Results

After the recent demonstration of the enzymatic nature of marinocine isolated from *M. mediterranea* as a lysine oxidase [19], we continued the characterization of this protein. We focused the study on its comparison to the FAD-dependent LαO as this was the most similar enzyme described in microorganisms showing antibacterial, cytotoxic and antitumor properties due to hydrogen peroxide formation [4,5]. Both enzymes inhibited *E. coli* growth using the antibiogram assay [19]. However, LαO was much more efficient than marinocine regarding H_2O_2 production. In terms of specific activities, LαO was about 700 times more active than marinocine [19]. This remarkable difference suggested different catalytic mechanisms for the oxidative deamination of L-lysine.

3.1. Kinetics approach

Both enzymes were compared using the fluorimetric assay for H_2O_2 and two spectrophotometric assays for α-keto acid and aldehydes determination. Fig. 1A, B and C show the kinetics of the 3 assays. The fluorimetric detection of H_2O_2 displayed a similar profile for both lysine oxidases (Fig. 1A). However, the kinetics of the spectrophotometric assays displayed remarkable differences. Concerning the semicarbazide assay for α-keto acids, the accumulation pattern of the semicarbazone derivative was distinct (Fig. 1B). LαO displayed a linear increase of A_{248}, supporting a linear accumulation of the semicarbazone from 2-keto-6-aminocaproic acid as previously reported [20]. Marinocine displayed an early saturation process, and after about 2–3 min the A_{248} nm remained constant. Taking into account the stability of marinocine [17], this pattern suggests that a different keto acid from L-lys was formed by marinocine, so that the semicarbazone formed did not accumulate. The most likely alternative for a different product from L-lys would be a ε-semialdehyde due to oxidative deamination of the ε-amine group.

The spectrophotometric assay for aldehyde determination using NAD^+ confirmed this alternative (Fig. 1C). Marinocine showed a linear rate of NADH formation, whereas LαO did not show NADH appearance. Thus, marinocine yielded a product which is able to be subsequently recognized as substrate of aldehyde dehydrogenase, but LαO did not. In sum, both enzymes would form different products from L-lys, marinocine releasing an ε-semialdehyde whereas LαO an α-keto acid.

Fig. 2A and B shows the correlation between enzyme amount (LαO and marinocine) and initial rates using the fluorimetric detection of released hydrogen peroxide and the spectrophotometric detection of the semicarbazone derivative. LαO displayed a good correlation between both methods (Fig. 2A), although marinocine showed small reaction rate for low amount of enzymes in the semicarbazide spectrophotometric method (Fig. 2B). This might reflect that the peroxidase-catalysed coupled reaction of Amplex red with H_2O_2 is very fast, but the non-enzymatic coupled reaction of semicarbazide with the keto-acid formed from L-lys is slower and different between both enzymes.

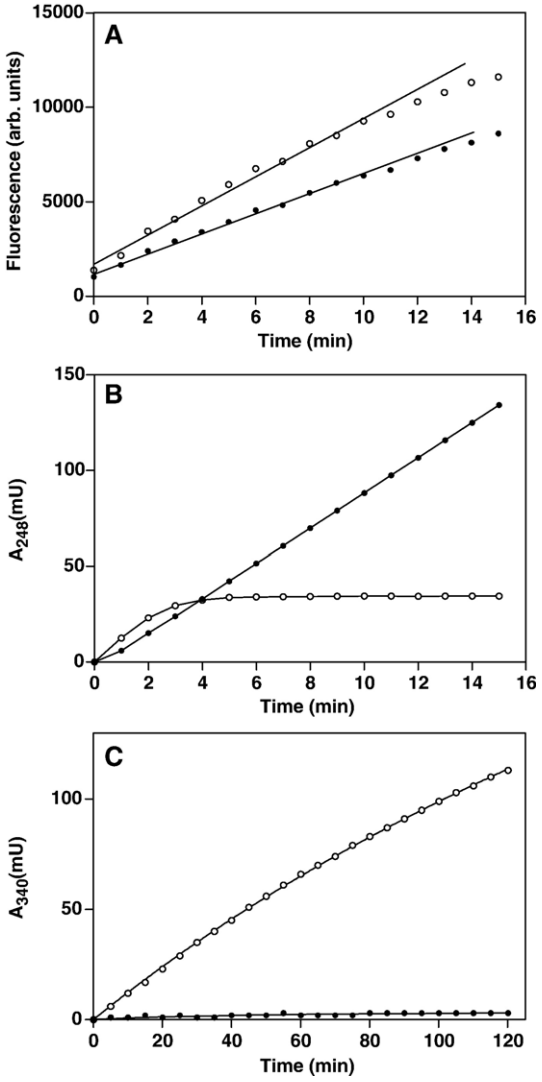

Fig. 1. Kinetics of reactions for LαO (○) and marinocine (●) acting on L-lys in 3 different assays for product determination. (A) Fluorimetric assay for H_2O_2 determination; (B) spectrophotometric assay using semicarbazide to trap the α-keto acid produced after L-lys deamination: LαO causes a linear accumulation of the product, but marinocine shows a rapid saturation process; (C) spectrophotometric assay for aldehyde determination. LαO did not generate NADH, whereas marinocine did it. Note that the amounts of enzyme used in panel A (0.1 mU/ml and 8 U/ml respectively) is 8 times less than in panels B and C (0.8 mU/ml and 40 U/ml) due to the higher sensitivity of the fluorimetric assay. It should be also taken into account that the units are not comparable, as LαO is expressed in IU and marinocine units are defined in reference to the diameter of antibiograms [17].

3.2. Characterization of the reaction catalysed by marinocine

To characterize the reaction products of L-lysine with LαO and marinocine, the assay mixtures were examined by TLC as described for LαO [4] (Fig. 3). The disappearance of the L-lysine spots in samples incubated with marinocine and LαO (Fig. 3) demonstrates the transformation of this amino acid by both enzymes. However, the reaction products formed were clearly different. As expected, LαO yields 5-aminovaleric acid (weak spot of this acid due to the poor reaction of this δ-amino acid with ninhidrin) by oxidative decarboxylation in the absence

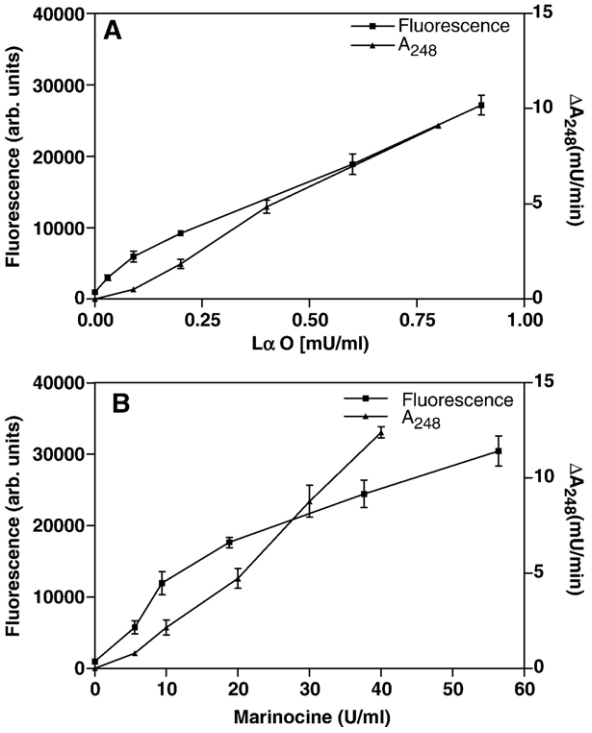

Fig. 2. Comparison between the Amplex red (fluorimetric, ■) and semicarbazide (spectrophotometric, ▲) methods for measuring LαO and marinocine activity. Panels A and B show the correlation between initial reaction rates and the amount of both oxidases. To facilitate comparison, data of both assays are expressed as increase of fluorescence (left axis) and absorbance units (right) per minute between 30 and 90 s of reaction. Enzymatic units are differently defined (see Materials and methods).

of catalase and Δ^1-piperideine-2-carboxylate in its presence. Catalase is used because in its absence, the immediate keto product after L-lysine deamination undergoes further oxidation due to the hydrogen peroxide formed [6], but in its presence the peroxide is rapidly decomposed and the reaction favoured for those products is an intramolecular cyclation to carboxy-Δ^1-piperideines (Fig. 4, upper part).

The nature of the reaction products formed by marinocine supports an oxidative deamination of the ε-amino group. Besides some low R_f unidentified hydrophilic products, two spots correspond to the main products formed depending on the presence or absence of catalase. The first one (spot A, lane 3) only appears in the absence of catalase and it has the same R_f that standard 2-aminoadipic. This acid can be formed by the peroxide-dependent oxidation of the 6-semialdehyde-2-aminoadipic acid. The second product formed (spots B and C) shows an R_f very similar to pipecolic acid and a little bit higher than the R_f of Δ^1-piperideine-2-carboxylate formed in the LαO mixtures (lane 6). In agreement with the likely intramolecular cyclation of the ε-semialdehyde, it should be Δ^1-piperideine-6-carboxylate (Fig. 4, lower part).

3.3. Substrate specificity

Substrate affinity of marinocine was studied using the fluorimetric assay. Marinocine was very specific for the L-isomer of lysine, showing a very high affinity for this substrate (Km = 2.8 ± 0.9 μM) and inhibition by excess of substrate for concentrations higher than 0.5 mM. The affinity for some other alternative substrates was much lower. Up to 10 μM, none alternative substrate produced measurable activity. Table 1 shows the percentage of activity for those substrates at concentrations ranging from 25 to 200 μM in comparison to the control activity for 5 μM L-lysine.

α-*N*-acetyl-L-Lys was a good substrate for marinocine indicating that the amine group in position alpha is not oxidized by this enzyme. On the contrary, ε-*N*-acetyl-L-lys was not a substrate. This pointed out that the modification of the ε-group abolished marinocine activity, supporting that this enzyme could catalyze the ε-deamination of L-lys. Other substrates with lower affinity were L-orn, D-lys and 5-hydroxy-L-lys in that order. This gives information about the great importance of factors such as the appropriate distance between the amino and carboxyl groups in L-lys (comparison to the shorter L-orn), the stereospecificity (comparison to D-lys) and the negative effects of the introduction of an hydroxy group adjacent to ε-amino group on the side chain of lysine (comparison to 5-hydroxy-L-Lys). Finally, other compounds with structural similarity to L-lys, such as the tetrapeptide LSKL, amino acids such as arginine and p-amino-L-phenylalanine, the amines putrescine, cadaverine and 1,6-hexanodiamine and the 5-aminovaleric, 6-aminocaproic, 2-aminoadipic and 2,7-diaminopimelic acids were not substrates of the enzyme (the residual activity on that tetrapeptide was 2.7%, and all others were lower than <0.5% in comparison to the control using L-lys, Table 1). This behaviour is clearly different from the data described for LαO and Lox.

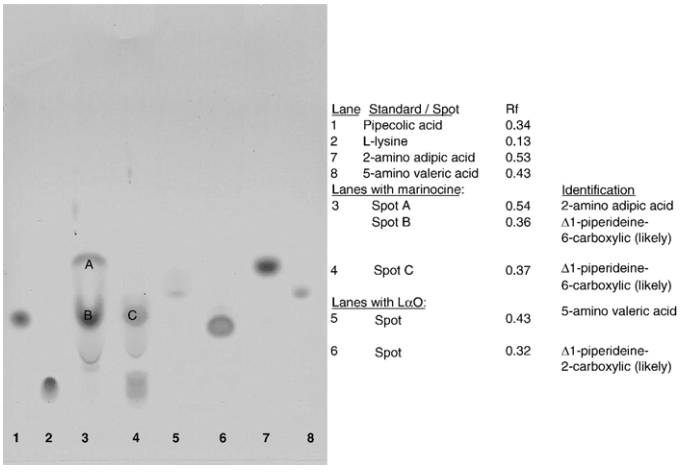

Fig. 3. TLC of some commercial standard compounds and the reaction mixtures of L-lysine incubated 12 h with LαO or marinocine in the absence or the presence of catalase. Lanes 1, 2, 7 and 8: pipecolic acid, L-lysine, 2-aminoadipic acid and 5-aminovaleric acid respectively. Lanes 3 and 4, reaction products after overnight incubation of marinocine with lysine in the absence (3) or presence (4) of catalase 0.1 mg/ml. Lanes 5 and 6, similar incubations with LαO in the absence (5) or presence (6) of catalase R_f of the standards and possible identified compounds are indicated.

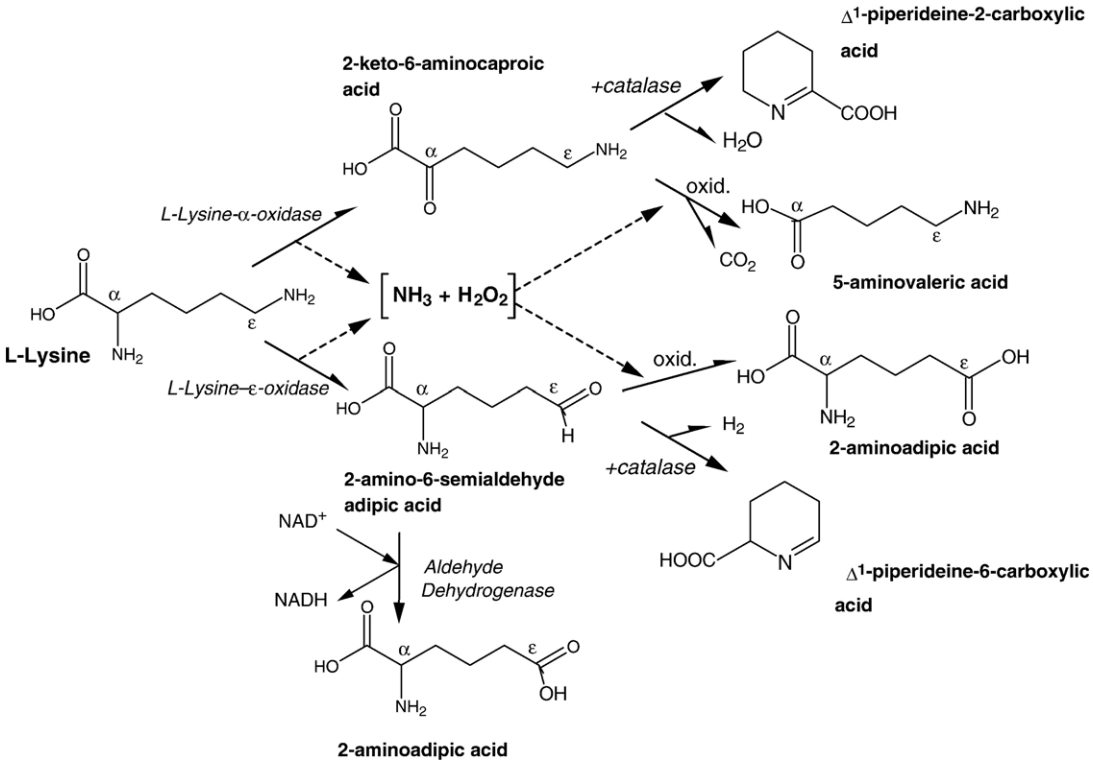

Fig. 4. Proposal of the reaction products after enzymatic action on L-lysine according to the TLC. Upper part: LαO yields 2-keto-6-aminocaproic acid. The formed H_2O_2 causes the ulterior oxidative decarboxylation of this acid to 5-aminovaleric, but in the presence of catalase it undergoes an intramolecular cyclation to Δ^1-piperideine-2-carboxylic acid. Lower part: Marinocine yields 6-semialdehyde-2-aminoadipic acid. Similar to the upper part, this acid would be oxidized to 2-aminoadipic by H_2O_2, but the addition of catalase favours its intramolecular cyclation to Δ^1-piperideine-6-carboxylic acid. Those Δ-piperideine carboxylic acids show a similar R_f to pipecolic acid, the most similar available standard. In relation to the semicarbazide assay, it can be also noted that the semicarbazones obtained of both enzymes is different, since LαO yields an α-keto acid but marinocine yields an ε-semialdehyde acid. Applied to the aldehyde dehydrogenase/NAD^+ assay, only the product formed by marinocine can be substrate of that enzyme with the appearance of reduced NADH.

3.4. Inhibitors

Inhibitors of marinocine activity were also studied. βAPN, cadaverine and 6-aminocaproic acid were good inhibitors (Table 1). 5-aminovaleric acid, aminoguanidine and amiloride showed a weaker but noticeable inhibition. Finally, semicarbazide, ε-*N*-acetyl-L-Lys, putrescine (1,4-diaminobutane), 1,6-diaminohexane, 1,7-diaminoheptane, benzylamine, gabaculine, 2-aminoadipate and 2,7-diaminopimelate did not inhibit marinocine. It is interesting to note that cadaverine and 6-aminocaproate were not substrates but they acted as competitive inhibitors. These results illustrate the importance of the appropriate distance between both amino groups for active site interaction and catalysis, since shorter (putrescine) and longer structures (1,6-diaminohexane, 1,7-diaminoheptane) were not substrates or inhibitors. Although some amine oxidases show the similar features [11], there are also clear differences with those oxidases. Thus, the distance between the amino and carboxyl groups is also crucial for marinocine, as 6-aminocaproic acid inhibits more than 5-aminovaleric acid.

βAPN is a well-known inhibitor of mammalian Lox, causing osteolathyrism [22,23]. This agent reacts with the active site of LTQ-containing enzymes causing a time-dependent inactivation. Fig. 5 shows the kinetics of inhibition of marinocine by βAPN and its shorter analogue AAN. βAPN was very effective at 50 μM, but AAN only showed a weak inhibition at higher concentrations (200 μM). According to the flavin-nature of LαO, neither 200 μM βAPN nor AAN inhibited this enzyme (data not shown).

3.5. Spectral properties

UV-Vis spectra of LαO and marinocine showed different profiles, and the FAD-associated peaks at 388 and 466 nm observed in LαO were not observed in marinocine (Fig. 6). Moreover, incubation of marinocine with a number of agents (NAD(P)H, FAD, piridoxal phosphate, riboflavine) did not produce any significant change in its antibacterial and lysine oxidase activities, indicating that this enzyme does not depend on FAD or any of these cofactors. The absence of FAD is particularly important to distinguish marinocine from LαO and LAO, as this is the cofactor found in those enzymes [4,6].

4. Discussion

We have recently shown that marinocine is a protein with antibacterial properties synthesized by the marine bacterium *M. mediterranea* [17] and that the H_2O_2 production of from L-lys deamination is responsible for the bactericide effect [19]. Since LαO from the fungi *T. viride* has been described with

Table 1
Substrate specificity and inhibition of marinocine by L-lysine analogues and some amino oxidase inhibitors

Putative substrate/inhibitor	Concentration as substrate (μM)	Activity as substrate (%)[a]	Inhibition (%)[b]
L-Lysine	5	100	0
α-*N*-acetyl-L-Lys	25	91.9	n.d.
L-Ornithine	25	15.1	n.d.
D-Lysine	200	3.2	n.d.
LSKL tetrapeptide	25	2.7	n.d.
5-hydroxy-L-Lys	25	1.9	n.d.
ε-*N*-acetyl-L-Lys	25	1.9	1
L-Arginine	200	<0.5	0
p-Amino-L-phenylalanine	200	<0.5	n.d.
Diaminopimelic acid	200	<0.5	0
6-Aminocaproic acid	200	<0.5	72
2-Aminoadipic acid	200	<0.5	0
5-Aminovaleric acid	200	<0.5	30
Putrescine	200	<0.5	0
Cadaverine	200	<0.5	74
1,6-diaminohexane	200	<0.5	0
1,7-diaminoheptane	200	<0.5	0
Benzylamine	200	<0.5	0
β-Aminopropionitrile		n.d.	91
Aminoacetonitrile		n.d.	4
Semicarbazide		n.d.	0
Aminoguanidine		n.d.	23
Amiloride		n.d.	25
Gabaculine		n.d.	0

[a] Activity of Lod with the putative substrates at the indicated concentrations. These values are expressed as percentage on respect to control reaction using 5 μM L-lys as substrate and the fluorimetric hydrogen peroxide assay with Amplex red. All values are the mean of at least two determinations. n.d. not determined.

[b] Percentages of inhibition by the presence of 100 μM of the tested compound in comparison to the standard assay using 5 μM L-Lys as substrate. Inhibitory effect lower than 0.5% was considered not significant, note the 1:20 ratio of substrate:inhibitor concentrations.

the same properties and antimicrobial applications [5], we continued the characterization of marinocine by comparing our enzyme with the fungal LαO. The fluorimetric measurement of H_2O_2 or by antibiogram had previously shown that the pattern of both enzymes was similar, although the specific activity of LαO was much higher than of marinocine [19].

However, the comparison of both enzymes by spectrophotometric assays provided evidence that aside from H_2O_2, other products formed by marinocine are different from those formed by LαO. The semicarbazone formed after marinocine action on L-lys was unstable and different from that one formed by conjugation of the reaction product of LαO, 2-keto-6-aminocaproic acid, with semicarbazide. In addition, the aldehyde dehydrogenase/NAD^+ assay showed that marinocine yielded an aldehyde rather than an α-keto acid. The last method showed poor sensitivity and it needed longer incubation times to detect NADH formation accurately, very likely due to the low affinity of aldehyde dehydrogenase to 6-semialdehyde 2-aminoadipic acid. Control experiments in our lab showed that aldehyde dehydrogenase had good affinity for small aldehydes, such as acetaldehyde, but low affinity for long-chain aldehydes such as glutaraldehyde (data not shown).

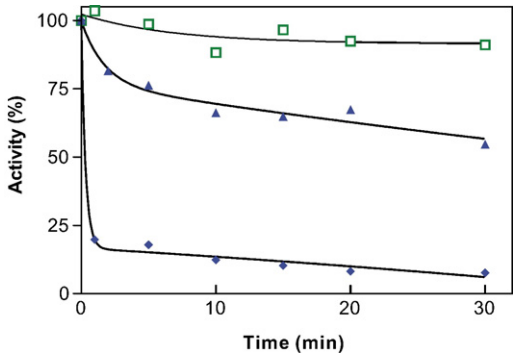

Fig. 5. Time-dependent inactivation of marinocine by incubation with βAPN and AAN. Residual activity (%, fluorimetric assay) after preincubation of marinocine (0.52 U) with 20 μM βAPN (▲) and 50 μM (◆) or (□) AAN 200 μM. LαO was not affected by these agents at concentrations 200 μM. Antibiogram assays were well correlated with these data (not shown).

Characterization of the reaction products by TLC confirmed the differences between LαO and marinocine. LαO leads to the formation of 5-aminovaleric acid [4] whereas marinocine leads to the formation of 2-aminoadipic acid. Data obtained in the absence or the presence of catalase sustain the reactions displayed in Fig. 4. Accordingly, marinocine is a novel type of lysine oxidase. We now propose to rename marinocine, suggested before this enzymatic activity was characterized [17], into lysine-ε-oxidase. Lod might also be used according to the name given to the cloned gene [19].

To prove its novel nature, the enzymatic characteristics of Lod should be distinguished from other enzymes involved in lysine transformation. To this purpose, the characteristics of two different groups of enzymes have been compared. The first group of enzymes transforms L-lys into 2-aminoadipic acid. The second group catalyzes the L-lys oxidative deamination releasing H_2O_2.

The enzymes catalyzing 2-aminoadipic acid formation are L-lysine-ε-aminotransferase (lat, E.C. 2.6.1.36), a piridoxal phosphate-dependent enzyme involved in the first step in the biosynthesis of cephalosporins in actinomycetes [25], and four saccharopine dehydrogenases (SDH, E.C. 1.5.1.*x*, *x*=7 to 10)

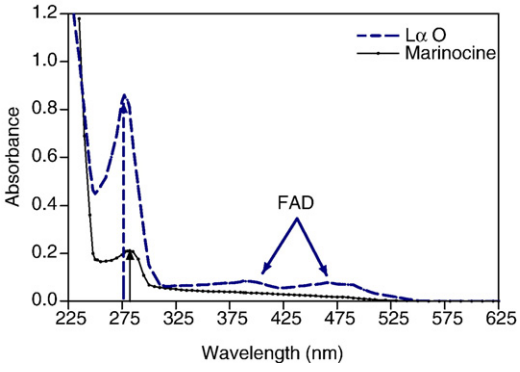

Fig. 6. UV-Vis spectra of LαO and marinocine. The UV peak of LαO is far more prominent than that of marinocine. More important, the small FAD-associated peaks in the visible region are well observed in LαO but are not in marinocine.

involved in the catabolism of L-lys in different types of cells. Lat belongs to the GABA transaminase family, showing good affinity for L-lys and also L-orn and being inhibited by gabaculine [26]. However, marinocine does not depend on piridoxal phosphate; its affinity to L-orn is very low and is not inhibited by gabaculine. Concerning the four SDHs, all depend on 2-ketoglutarate but do not release H_2O_2, in clear distinction to marinocine. In addition, marinocine does not depend on NAD or NADP, and it does not form saccharopine as intermediate.

In relation to the second group of enzymes catalyzing the oxidative deamination of L-lys and the formation of H_2O_2, marinocine was compared to LαO and Lox. LαO is clearly different as it does not form 6-semialdehyde 2-aminoadipic acid. Lox acts on free diamines and lysine-containing peptides, but not on free L-lys. In addition to these crucial differences with those lysine oxidases, other data related to substrate specificity, inhibition pattern and molecular properties reinforce that marinocine is a novel type of lysine oxidase.

Concerning substrate specificity, the differences of Lod with LαO and Lox are clear. The most evident one is illustrated by the *N*-acetyl derivatives of lysine as alternative substrates. ε-*N*-acetyl L-Lysine is not substrate of Lod. However, α-*N*-acetyl L-Lysine is a good substrate, the best one aside L-lysine, indicating that the importance of the free α-amino group in the catalysis is poor. This pattern is completely opposite for LαO [4]. Lox has very weak affinity for free lysine and both acetylated derivatives [13,21].

The differences can be reinforced with other data about substrate specificity. Lod is very specific for free L-lys. LαO is also rather specific on L-lys, but similar amino acids are also relatively good substrates, e.g. 5-hydroxy-L-lysine [4], whereas Lod shows a residual activity around 2% on this compound. LAO from *Aplysia californica* oxidizes L-lys and L-arg, whereas Lod only the first one [6]. The stereospecificity of Lod for the D-/L-lys pair is also higher than the stereospecificity showed by LαO.

Lox is active only on neutral lysine-containing peptides, as the LSKL, but no free L-lys [13]. On the contrary, these peptides are not substrates for Lod and LαO. Negative carboxylic charge at the α carbon of free L-lys or adjacent negative charges (Asp or Glu residues) in lysine-containing peptides are highly unfavourable for optimal interaction with the Lox active site. Similar to this mammalian enzyme, Lox from *Pichia pastoris* shows much more affinity on endopeptidyl lysine residues and diamines than on free L-lys and L-orn [10]. Typical diamine or amino oxidase substrates, such as 1,5-diaminopentane, n-hexylamine and benzylamine, are very good substrates of mammalian and *Pichia pastoris* Lox, [10]. However, none of those are substrates of Lod or LαO.

Other inhibitors are informative to explore the nature of the cofactor at the lysine oxidase active site and to facilitate classification. According to its inhibition profile, Lox belongs to the family of the SSAO [11,15,16] as it is considered a special type of amino oxidase. Besides semicarbazide, other very good inhibitors for SSAO amino oxidases are aminoguanidine, hydroxylamine and amiloride. However, all these agents do not have any effect on LαO. Lod is not inhibited at all by semicarbazide, but aminoguanidine and amiloride have a noticeable inhibitory effect (Table 1). Hydroxylamine could not be tested due to its interference with Amplex red in the assay media. Thus, regarding typical SSAO inhibitors, marinocine displays a different inhibition profile from Lox and LαO.

Aminonitriles inhibit TDQC-containing enzymes but do not FAD-containing enzymes [12,23]. Marinocine is inactivated by βAPN in a time-dependent manner, similarly to Lox and differently to LαO. Inactivation data with βAPN suggest the existence of a TDQC as the prosthetic group of marinocine. However, the inhibition pattern by the shorter analogue AAN raises some doubts about the exact nature of the cofactor in Lod. AAN seems to be a stronger inhibitor than βAPN in LTQ-containing enzymes as Lox [23], but it is not so for Lod. In the same way, semicarbazide inhibits LTQ-containing enzymes, whereas Lod is not inhibited by this agent yet.

Concerning spectral properties, LαO is a FAD-enzyme, but Lod is not. The UV peak of LαO is located at 277 nm, in agreement with the presence of FAD in that enzyme, but the maximum of the Lod peak was observed at 282 nm. More important, its UV-Vis spectra did not show the peaks of 388 and 466 nm associated with FAD even when the protein concentration was a bit higher in the Lod purified preparation than in LαO from *T. viride*. The peak at 282 nm is compatible with the spectra of LTQ-containing enzymes, such as Lox [24] and with the spectrum of model LTQ compounds [27], but the definitive nature of the TDQC in Lod should wait for new studies. Inhibition data obtained with βAPN and AAN suggest the presence of that type of cofactor, but its complete elucidation among the 5 different variants [12] needs further studies. It does not appear that topaquinone is this cofactor as it is not inhibited by βAPN. It also does not appear to be the case for LTQ since AAN should be more effective as inhibitor than βAPN, as it was reported in Lox [23].

In turn, preliminary experiments aimed at characterizing Lod as a copper enzyme were unsuccessful. Incubations with bathocuproine, biquinoline ascorbic acid and EDTA did not significantly change its activity. Although these data do not allow establishing the presence of copper, this possibility cannot be ruled out, since copper is frequently not removed from the active site of similar enzymes under similar mild non-denaturant conditions. For instance, Lox needed the presence of high concentrations of urea to remove copper from the active site by copper chelators [24]. Furthermore, the role of copper in the catalytic cycle of Lox is doubtful [27,28]. Models of copper and o-phenolic compounds exhibited amine oxidase-like activity [29], and recently, it has been demonstrated that copper is needed for the self-processing formation of LTQ cofactor in Lox [30].

Concerning possible similarities at the amino acid sequences of lysine oxidases, most SSAO enzymes, including Lox from *Pichia pastoris*, contain some characteristic common motifs in the C-terminal half of the protein. Mainly, the conserved signature motif NY(D/E)Y at the active site, where the first tyrosine is the precursor of the topaquinone

Table 2
Comparison of the essential residues in some selected TDQC-containing SSAO amino oxidases (including *Pichia pastoris* Lox), marinocine and lysine oxidases

SSAO consensus:	YxD ~ x(60) H	~x(30)	N**Y**DY	~x(50)	HxH
Marinocine (Lod)	^{479}YFD	^{544}H	^{578}YYS**Y**		^{603}HSH
Mammalian Lox consensus:	HxHxH ~x(20)	**K** ~x(30)	CYDT**Y**		
Human Lox, isoform 4	HxHxH ~x(20)	**K** ~x(30)	CWDT**Y**		
Drosophila Lox 1 & 2	HxHxH ~x(20)	**K** ~x(30)	C(A/S)DT**Y**		

Copper-binding histidines are in grey background, the cross-linked lysyl residue in LTQ-containing enzymes in bold, and the tyrosyl residue oxidized to quinone in bold higher size.

[10,15]. There are also 3 conserved H which bind the copper ion, one HxH motif 50 residues towards the C-terminal from the tyrosine-derived cofactor and a third H around 20–30 residues to the N-terminal side from the cofactor. To complete the essential conserved motifs, an YxD motif is found around 60 residues before that H, where Y is the gate for substrate access and D has been identified as the active site base (Table 2).

Mammalian Lox are LTQ proteins and they contain a similar arrangement. The consensus for the cofactor formation is also at the C-terminal half and one essential lysyl residue is needed for the LTQ formation. In these enzymes, there is no indication of the residues involved in the gate for substrate access. Interestingly, the 3 H binding-copper residues are close together in an HxHxH motif, but prior to the lysyl residue involved in the LTQ group and the motif where the tyrosine to be converted to quinone and cross-linked with the above mentioned lysine is located (Table 2). That tyrosine is the second one in the motif in most Lox, or just the only one existing in some species, as *Drosophila* [30] and human isoenzyme 4 [31].

Lod clearly shows more similarities to the SSAO signature motifs than to Lox (Table 2), e.g. related to the histidine disposition to bind copper. Interestingly the essential tetrapeptide for cofactor formation is not identical to any other enzyme, as ^{578}YYSY^{581} has 3 tyrosine residues, so it would be possible to form a tyrosine derived quinone at a position similar to SSAO but also similar to Lox. It is not possible to indicate the existence of a possible lysyl residue to cross-link, as there are no such residues at a distance similar to that one found in mammalian Lox. With the current data, it is not possible to establish which tyrosyl residue is oxidized and further proteomic experiments will be needed to establish the exact nature and position of the Lod cofactor. To finish these comments on amino acid similarities at the active site of lysine oxidases, LαO has not been cloned and hence comparison in the amino acid motifs cannot be performed, but it is obvious that the sequence should be very different, since the cofactor is FAD. Sequenced amino oxidases containing FAD show that the prosthetic group was found to be in an extended conformation buried in the protein core, and no specific amino acid motifs are involved in the binding.

In conclusion, Lod shows a very high affinity and specificity for L-lys. Any modification in the structure of this substrate greatly decreases that affinity. The three charged hydrophilic groups in the molecule, the α-carboxyl, α-amino and ε-amino, must be at the appropriate distances. Spatial orientation seems to be essential for substrate recognition, although only the ε-amino group is deaminated. Note that α-carboxyl is essential for enzymatic action, but is not for binding to the active site, as cadaverine is not a substrate but can act as a competitive inhibitor. This feature clearly distinguishes Lod from the other lysine oxidases. Structure–function correlations are implied in the formation of the enzyme–substrate complex establishing clear differences with other well-known lysine-related oxidases, LαO and Lox.

Marinocine, the protein product from gene *lod* [19], is the first described enzyme which catalyses the oxidative transformation of free L-lysine to the 6-semialdehyde 2-aminoapidic acid releasing H_2O_2. Its catalytic and molecular properties distinguish this enzyme from any other involved in lysine transformation so far included in the enzyme nomenclature list. We propose that this enzyme should be named lysine-ε-oxidase or lysine-6-oxidase, and a new number should be assigned by the Enzyme Commission. Communication to the IUBMB Biochemical Nomenclature and Enzyme database is underway to include it in the enzyme list with the number E.C. 1.4.3.X, according to the instructions of databanks (www.expasy.ch/enzyme/enz_new_form.html).

Acknowledgements

This work was supported by grant BIO2004-4803 from the Spanish MEC. We are grateful to James Leichter from SIO, La Jolla, for his useful editing review and suggestions. D.G. dedicates this work in memoriam of his sister, Laura.

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