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Interaction of L-lysine and soluble elastin with the semicarbazide-sensitive amine oxidase in the context of its vascular-adhesion and tissue maturation functions

Aldo Olivieri[1], Jeff O'Sullivan[2], Luis Raimon Alvarez Fortuny[3], Itziar Larrauri Vives[3] and Keith F. Tipton[1]

1. School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland
2. Dublin Dental School and Hospital, Trinity College, Dublin 2, Ireland
3. Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Spain

Corresponding author: Aldo Olivieri, oliviera@tcd.ie

Abstract

The copper-containing quinoenzyme semicarbazide-sensitive amine oxidase (EC 1.4.3.21; SSAO) is a multifunctional protein. In some tissues, such as the endothelium, it also acts as vascular-adhesion protein 1 (VAP-1), which is involved in inflammatory responses and in the chemotaxis of leukocytes. Earlier work had suggested that lysine might function as a recognition molecule for SSAO/VAP-1. The present work reports the kinetics of the interaction of L-lysine and some of its derivatives with SSAO. Binding was shown to be saturable, time-dependent but reversible and to cause uncompetitive inhibition with respect to the amine substrate. It was also specific, since D-lysine, L-lysine ethyl ester and ε-acetyl-L-lysine, for example, did not bind to the enzyme. The lysine-rich protein soluble elastin bound to the enzyme relatively tightly, which may have relevance to the reported roles of SSAO in maintaining the extra-cellular matrix (ECM) and in the maturation of elastin. Our data show that lysyl residues are not oxidized by SSAO, but they bind tightly to the enzyme in the presence of hydrogen peroxide. This suggests that binding *in vivo* of SSAO to lysyl residues in physiological targets might be regulated in the presence of H_2O_2, formed during the oxidation of a physiological SSAO substrate, yet to be identified.

1. INTRODUCTION

Semicarbazide-sensitive amine oxidase (SSAO) is a common name for primary-amine oxidase (EC 1.4.3.21), which was previously classified with a group of other enzymes as EC 1.4.3.6 [1]. It is a copper-containing quinoprotein that catalyses the oxidative deamination of endogenous and exogenous primary amines, according to the overall reaction:

$$RCH_2NH_2 + O_2 + H_2O \xrightarrow{\text{SSAO}} RCHO + NH_3 + H_2O_2$$

SSAO is known to be associated with cell membranes and blood vessels in mammalian species [2]. SSAO is a large, dimeric, type II transmembrane protein, with a very small intracellular domain and a large glycosylated extracellular domain containing the catalytic centre [3]. A soluble form of the enzyme also exists, circulating in plasma , which appears to be the result of proteolytic cleavage of membrane bound SSAO and it is likely that several tissues may contribute [4,5]. In humans SSAO is present in most organs and tissues, especially in vascular endothelial and smooth muscle cells, adipocytes and the umbilical cord, although it is absent from brain [6]. In addition to its amine oxidase functions, which include activities towards a number of endogenous and xenobiotic amines [7] SSAO appears to be involved in several different cellular processes [8]. In some tissues SSAO functions as a vascular adhesion protein, VAP-1, that mediates the slow rolling and adhesion of lymphocytes to endothelial cells [9]. The nature of the target site on lymphocytes to which endothelial SSAO/VAP-1 binds is unknown. Cell-surface amino sugars might be possible candidates since they have been shown to interact with SSAO [10] in a process that required hydrogen peroxide, with that formed during substrate oxidation being most effective. However, cell-surface amino sugars are generally *N*-acetylated and the free amine group has been shown to be necessary for binding [11]. In preliminary studies, we have shown L-lysine to behave in a similar manner to the amino sugars, inhibiting SSAO/VAP-1 in the presence of the H_2O_2 generated during benzylamine oxidation [12]. The present work was designed to extend those observations, using kinetic and binding assays. The behaviour of some lysine derivatives and lysine containing peptides was also investigated, in particular that of the lysine-rich protein soluble elastin. Cross-link synthesis in elastin and in collagen involves oxidative deamination of lysine residues by another copper-containing amine oxidase, lysyl oxidase (E.C. 1.4.3.13). SSAO, although a copper containing enzyme and having a TOPA-quinone as a cofactor, is distinct from lysyl oxidase as SSAO is extractable with detergents such as Triton X-100, has a molecular mass near 180 kDa, and it has not been reported yet to act on the soluble precursor of elastin, tropoelastin [13]. In a study by Langford *et al*. [14] rats were treated acutely or chronically with either semicarbazide or the more potent and selective SSAO inhibitors, such as allylamine derivatives. Treatment with these compounds produced acute and chronic lowering of SSAO activity in aorta and lung with little effect on the activity of lysyl oxidase, along with lesions consisting of striking disorganization of elastin architecture within the aortic media. This suggested a role for SSAO in connective tissue matrix development and maintenance, and specifically in the development of mature elastin [14]. cDNA studies on recombinant tropoelastin

confirmed that the structure of tropoelastin consists of alternating hydrophobic and crosslinking domains. In the crosslinking regions, the lysine residues usually occur in pairs, or triplets (near the central part of the molecule) among clusters of alanine residues [15]. To better understand the mechanism through which SSAO is involved in the maturation of elastin, it was decided for the present work to undertake a series of kinetic and binding studies focused on the interactions between bovine plasma SSAO and commercially available soluble elastin.

2. MATERIALS AND METHODS

2.1 Materials

Bovine plasma SSAO was purified by an adaptation [16] of the chromatographic procedure of Wang et al. [17]. For binding assays a preparation of SSAO by the supplier BioVar Ltd, Yerevan, Armenia, was also used. L-lysine, poly-L-lysine hydrobromide (M_r range =1000 to 5000) and soluble elastin, purified from bovine neck ligament by the method of Partridge et al. [18,19], were purchased from Sigma-Aldrich. Amplex Red reagent was purchased from Invitrogen. Other enzymes and chemicals used in this study were obtained from Sigma-Aldrich.

2.2 Enzyme Assay procedures

All assays were performed at 37 ^{o}C. SSAO activity towards benzylamine was assayed by a modification of the direct spectrophotometric method of Tabor *et al*. (1954) [13]. The standard reaction mixture contained 200 mM potassium phosphate buffer, pH 7.2, and 0.010 mg/ml SSAO (final concentration). The reaction was initiated by the addition of the substrate benzylamine and the rate of change of absorbance, at 250 nm, was monitored, using a Cary 300-Bio spectrophotometer. The molar extinction coefficient for benzaldehyde is 13800 M^{-1} cm^{-1} [20].

The oxidation of methylamine was determined spectrophotometrically by coupling the formation of formaldehyde to the reduction of NAD^+ in the presence of formaldehyde dehydrogenase (FDH) [21]. The reaction mixture contained, in a total volume of 1 ml, 0.1 unit of formaldehyde dehydrogenase, 200 μM NAD^+, 20 mM methylamine and in 1 ml 200 mM potassium phosphate buffer, pH 7.2. The formation of NADH was followed on a Cary 300-Bio spectrophotometer at 340 nm.

The formation of H_2O_2 was determined spectrophotometrically [22] or fluorimetrically [23] For the spectrophotometric method a chromogenic solution was prepared as previously described [22]. Reactions were followed using a SpectraMax 340PC microplate reader at 498 nm. The molar extinction coefficient for the dye produced is 4654 M^{-1} cm^{-1}. The fluorimetric assays were run in a Spectramax Gemini EM Fluorimeter, at a constant temperature of 37 ^{o}C, in 96-well plates, each well containing 0.5 U/ml horseradish peroxidase and 50 μM Amplex Red, in 200 mM potassium phosphate buffer, pH 7.2 and a final assay volume of 200 μl. The wavelengths were set at 550 nm (excitation) and 585 nm (emission).

When L-lysine was assayed as potential SSAO substrate, by both the fluorimetric and the spectrophotometric H_2O_2 detection methods, six groups were assayed for each

4

run: one contained buffer plus SSAO (0.005, 0.010, 0.015 mg/ml, final concentration), a second contained buffer and L-lysine (0.25, 1, 2 and 4 mM), in group 3, 4 and 5 each of the three concentrations of the enzyme as indicated above were assayed in the presence of the four concentrations of lysine. The control contained 0.005 mg/ml SSAO and its substrate, benzylamine (1 mM). The detection limit for this assay has been reported to be about 5 nM H_2O_2 [23].

2.3 Inhibition studies

Reversibility of inhibition was assessed by dilution. The enzyme (protein concentration, 0.1 mg/ml) and the inhibitor were preincubated until inhibition was essentially complete (>90 %), using previous time-course experiments as a guide. 10 μl samples were then removed and diluted into the standard benzylamine assay mixture (final volume = 1 ml), to give a final protein concentration of 0.010 mg/ml, for activity determinations. A control sample was also taken through the same procedure where inhibitor was added to the corresponding final concentration, after dilution. A further test involved incubation of the enzyme (0.01 mg/ml) plus substrate (5 mM benzylamine) and 1 mM L-lysine and monitoring the reaction at 250 nm. After 60 minutes, when benzaldehyde production had essentially ceased. additional enzyme (0.01 mg / ml, final concentration) or substrate (5 mM, final concentration) was added. The progress of benzylamine oxidation was then further monitored.

To quantify the time dependence of the inhibition by L-lysine, the enzyme (0.010 mg/ml, final concentration) was preincubated at different times with the amino acid (0.5 mM), in 0.2 M phosphate buffer, at pH 7.4 and at 37 °C. Solutions of L-lysine and some of its derivatives were found to contain small, but variable, amounts of H_2O_2 determined, by the Amplex Red procedure, to be in the range of up to 1 μM. To avoid possible interference from this source, 0.001 units of catalase were added to the reaction mixtures for studies on the inhibition by L-lysine alone. The reactions were then started by the addition of 5 mM benzylamine and their progress was monitored at 250 nm for 15 min.

The effects of the products of the SSAO-catalysed reaction were studied by preincubating the enzyme (0.010 mg/ml, final concentration) for various times in 0.2 M phosphate buffer, 0.5 mM L-lysine and one of the following: 1 mM H_2O_2 (in the presence or in the absence of catalase, 0.1 units), 1 mM ammonium chloride or 1 mM benzaldehyde, before starting the assay with benzylamine (5 mM). When the effects of H_2O_2 and ammonium chloride were studied, the reaction was assayed by following the production of benzaldehyde at 250 nm. The high absorbance of the added benzaldehyde precluded the use of this assay when the effects of preincubation with that product were studied. In this case the formation of H_2O_2 was monitored by the coupled photometric assay, as described above. Controls in the absence of SSAO were used to ensure that L-lysine did not interfere with either detection system.

2.4 Kinetics of inhibition

The apparent value of K_i (the dissociation constant of the non-covalent complex) for the inhibition of SSAO by L-lysine was determined by two different approaches. The first involved analysis of the reaction progress curves, whereas in the second approach only the initial rates in the presence of 0.1 mM externally added H_2O_2 were considered. In the

5

first method, the value of K_i was obtained by fitting the reaction time courses, such as those shown in Fig. 1, to the model shown in Scheme 1, which may be described by the first-order process, shown in equation (1) [10, 24].

INSERT SCHEME 1 HERE

$$P_t = P_\infty (1 - exp^{-k'.t}) \qquad (1)$$

Where P_t is the product concentration at any time t, and P_∞ is the final product concentration when the reaction has ceased.

The relation between apparent first-order constant for loss of activity, k', and K_i will depend on the type of non-covalent interaction with the enzyme. If this is competitive with respect to the amine substrate, as implied by Scheme 1, the relationship will be:

$$k' = k\{1 + (K_i / I)[1 + (S / K_m)]\}^{-1} \qquad (2)$$

whereas uncompetitive inhibition will be described by the relationship:

$$k' = k\{1 + (K_i / I)[1 + (K_m / S)]\}^{-1} \qquad (3)$$

S and I represent the substrate and inhibitor concentrations, respectively. Non-linear regression, using the software GraphPad Prism (version 5.0) was used to determine k' and P_∞ and, since the value of K_m for benzylamine is known, from separate experiments, to be 1210 ± 180 μM, the values of k and K_i could be determined according to equations (2) or (3).

When the initial-rate approach was used, the enzyme activity was assayed in the presence of seven different concentrations of substrate (benzylamine; from 0.2 to 2 mM) and in the presence of 0.1 mM H_2O_2. Samples containing the concentrations of substrate indicated above were assayed in the presence of five different concentrations of L-lysine (0 to 1 mM). The initial rates of formation of benzaldehyde were then monitored at 250 nm. The data obtained from four different experiments were then fitted to the Michaelis-Menten equation by non-linear regession and the value of the value of K_i was determined from the dependence of the kinetic parameters on the inhibitor concentration. Where half-maximal inhibitory concentration (IC_{50}) values were used as a measure of inhibitor potency, these were determined from dose-response curves in which the values of enzyme activity were plotted against the logarithm of the molar concentration of the doses of inhibitor used. The resulting sigmoidal curves were fitted to a four-parametric model, with variable slope and the IC_{50} values, corresponding to the point of inflection of such curves, were obtained.

2.5 Fluorimetric assays for quantification of protein binding
The binding of ligands to SSAO was monitored by changes in the tryptophan fluorescence intensity, measured at emission and excitation wavelengths of 343 nm and 270 nm, respectively. Samples of SSAO (3.33 nm) in 0.1 M phosphate buffer, pH 7.4, were titrated with increasing concentrations of ligand, at 37 ^{0}C and with continuous

stirring. The relative fluorescence values, correspondent to $F_{SSAO\_Ligand}/F^0_{SSAO}$ (where F_{SSAO_Ligand} is the fluorescence of the SSAO-ligand complex and F^0_{SSAO} is the fluorescence of 3.33 nm SSAO in the absence of ligand), were then corrected by subtraction of background due to addition of ligand (if this contributed to the total fluorescence) and plotted as a function of the concentration of added ligand. The data were then fitted to to a hyperbolic binding curve by non-linear regresion to obtain an approximate values of the dissociation constant, K_d.

2.6 Fitting and statistical procedures

The program GraphPad Prism, version 5.0, was used for all curve-fitting procedures. Double-reciprocal plots are used for illustrative purposes only. Standard errors of mean were determined from at least 3 separate experiments.

3. RESULTS

3.1 Inhibition of SSAO by L-lysine

The presence of L-lysine during the oxidation of benzylamine (5 mM) resulted in a dose-dependent inhibition of SSAO activity (Fig.1a). Similar results were obtained with methylamine (20 mM) as the substrate. As shown in Fig.1b the inhibition was greatly reduced by the presence of 0.15 units/ml of catalase, suggesting the involvement of H_2O_2, produced during substrate oxidation, in the process.

The ability of L-lysine to act as a substrate for SSAO was assessed by incubation of the enzyme with the range of L-lysine concentrations used in the inhibition studies for periods of up to 2 h at 37 °C and determining H_2O_2 formation by the procedures described earlier. There was no detectable formation of this product, suggesting that, within the detection limits of the assay, L-lysine is not oxidised by SSAO.

3.2 Time dependence of the inhibition of SSAO by L-lysine and the effects of the products of the deamination of benzylamine on the inhibition

Preincubation of SSAO with L-lysine (0.5 mM), at 37 °C, before adding substrate showed low levels of inhibition in some, but not all, experiments. However this was abolished by the inclusion of catalase (0.001 units), which had no effects on its own, suggesting that the effect was due to the variable contamination with H_2O_2, discussed above. However, the inclusion of 1 mM hydrogen peroxide in the preincubation mixture resulted in essentially complete inhibition at all preincubation times, an effect that was prevented by the inclusion of catalase. Preincubation of SSAO with hydrogen peroxide (1 mM) on its own for 20 min did not affect the rates of oxidation of benzylamine, but there was some significant inhibition after 40 min (ca. 30 %, $p < 0.05$ with respect to controls), as shown in Fig. 2. As might be expected, this inhibition was prevented by the inclusion of catalase (not shown).

The other products of the of the SSAO-catalysed oxidation of benzylamine, ammonia, added as 1 mM NH_4Cl, and benzaldehyde (1 mM) had no significant effects

on the activity after preincubation with the enzyme, either in the absence or presence of L-lysine.

3.3 Reversibility of the inhibition of SSAO by L-lysine

The inhibition of SSAO by L-lysine after preincubation for 20 min, both in the presence and absence of H_2O_2 was found to be largely reversible by dilution. The SAO activity was restored to around 75 ± 5 %, compared to control, after dilution. After the enzyme and benzylamine had been incubated with L-lysine for up to 60 minutes, the addition of extra substrate did not reverse the inhibiton. Addition of fresh enzyme did, however, restore activity, which subsequently declined with time (data not shown).

3.4 Kinetics studies of the inhibition of SSAO by L-lysine.

Initial-rate determinations in the presence of 0.1 mM H_2O_2 showed that K_m/V_{max} for benzylamine was unaffected by the concentration of L-lysine (Fig. 3a) suggesting uncompetitive inhibition, where the inhibitor binds only to the complex formed between the enzyme and the substrate, or an intermediate product. The K_i value, obtained from the variation of K_m or V_{max} with L-lysine concentration was 166 ± 48 µM.

K_i values were also determined by analysis of reaction progress curves such as shown in Fig. 1a. By fitting the data to equation 1, first order rate constants were obtained to use in equation 3, from which a K_i value of 103 ± 14 µM for inhibition of benzylamine oxidation by L-lysine by uncompetitive inhibition (Fig. 3b).

3.5 Effects of the derivatives of L-lysine and of soluble elastin

D-lysine (Fig. 1a), L-lysine ethyl ester, tosyl-L-lysine ethyl ester, ε-acetyl-L-lysine and 6-aminocaproic acid all gave no significant inhibition at concentrations up to 1 mM, when added to the assay mixture, or in the presence or absence of 0.1 mM H_2O_2. The basic amino acid L-arginine (1 mM) was also without effect. 1 mM α-acetyl-L-lysine and poly-L-lysine in the range of 1-5 mM both gave a low level of inhibition (a maximum of ca. 20 %). Soluble elastin, was not oxidized by the enzyme, but inhibited SSAO with an IC_{50} of approximately 4.6 mg/ml, as shown in Fig.4. The major product contained in soluble elastin has a M_r of 70,000 [15], so 4.6 mg/ml corresponds to an IC_{50} of about 66 µM.

3.6 Binding assays

Addition of L-lysine caused a reduction of the tryptophan fluorescence of SSAO. Fig. **5** shows the relative decrease as a function of the concentration of L-lysine, in the presence of 0.1 mM H_2O_2 or 10^{-3} units/ml of catalase. L-lysine bound to SSAO in the presence of H_2O_2 with an apparent dissociation constant (K_d) of 0.10 ± 0.01 mM, whereas there was little binding in the presence of catalase (ca. 5 % reduction in fluorescence intensity compared to unbound SSAO). Addition of D-lysine, instead of L-lysine, had no effect on the tryptophan fluorescence, either in the presence or in the absence of H_2O_2 (data not shown). The stepwise titration of 3.33 nM SSAO with increasing concentrations of soluble elastin, in the concentration range from 0 up to 0.25 mg / ml (approximately 3.54 µM), is shown in Fig. 6. In the presence of 10^{-3} units/ml of catalase there was a non-hyperbolic decrease of fluorescence, suggesting that there was some non-specific binding of soluble elastin. Subtraction of the non-specific binding from the binding curve

obtained in the presence of H_2O_2 resulted in a hyperbolic curve with a K_d of 0.05 ± 0.02 mg /ml, corresponding to approximately 0.70 µM.

4. DISCUSSION

The endothelial-cell, membrane-bound, SSAO has been shown to mediate the adherence and migration of lymphocytes into tissue [9] and the plasma-soluble form increases in the presence of some inflammatory conditions [6] The nature of the target site on lymphocytes to which endothelial SSAO/VAP-1 binds is currently unknown, although, as discussed earlier, amino sugars and lysine-containing proteins have been suggested as possible candidates. Preincubation of the enzyme with free L-lysine plus H_2O_2 was found to result in time-dependent decreases in the initial rates of benzylamine oxidation. Furthermore this inhibition was prevented by the presence of catalase. The other products of the amine-oxidase catalysed oxidative deamination of benzylamine reaction, benzaldehyde and ammonia, had no significant effects on the inhibition of L-lysine, when present at concentrations very much higher than those that would be formed during the enzyme-catalysed reaction.

In the presence of H_2O_2 L-lysine binding, as assessed by inhibition of substrate oxidation and perturbation of tryptophan fluorescence, follows saturation kinetics and appears to be specific since D-lysine neither bound nor inhibited. Wang et al. [17] reported L- and D-lysine to be very poor substrates for bovine plasma SSAO. However, under the same conditions used in that work, but with a more sensitive assay procedure, we could not detect any significant oxidation of L-lysine. L-lysine ethyl ester, tosyl-L-lysine ethyl ester, and 6-aminocaproic acid were not inhibitors and neither was the basic amino acid, L-arginine. However, α-N-acetyl-L-lysine was an inhibitor, albeit a poor one, suggesting that a free ε-amine group might be important for the interaction. Poly-L-lysine was also found to be a weak inhibitor of SSAO ($20 \pm 1\%$ at 5 mM).

The kinetic studies indicated that L-lysine behaved as an uncompetitive inhibitor with respect to benzylamine, a type of inhibition where the inhibitor does not bind to the free enzyme but only to an intermediate complex formed during the reaction. SSAO from this source has been shown to operate through a double-displacement, or ping-pong, mechanism in which amine oxidation results in the release of aldehyde product and the formation of a reduced form of the enzyme, which is subsequently reoxidised by oxygen and the formation of H_2O_2 [25,26]. In such a system an inhibitor that bound to the free reduced form of the enzyme might be uncompetitive with respect to the amine substrate, however, that would not account for the requirement for H_2O_2. In this case it appears that the complex formed includes hydrogen peroxide or a related species is involved. It appears that several activated oxygen species are formed between the binding of O_2 to the reduced enzyme and the release of H_2O_2 [25] and the possibility that L-lysine binds to one of these, rather than the enzyme.H_2O_2 complex cannot be excluded, since it is not known whether the high concentrations of externally added H_2O_2 might reverse the steps involved. This may explain why the concentrations of added H_2O_2 necessary for significant inhibition when SSAO was preincubated with L-lysine were very much higher than those formed during the oxidation of benzylamine. However, poor accessibility of the site at which H_2O_2 is bound [27] may also contribute.

Hydrogen peroxide is also necessary for the binding of amino sugars to SSAO

[10,11] and others have suggested it might mediate the process of vascular adhesion [3,8]. It has been proposed that H_2O_2 may induce conformational changes in SSAO due to sulfydryl-group oxidation to form a vicinal disulfide bond, and that this may play an important role in modulating its actions [28]. However, the amount of H_2O_2 generated during the oxidation of benzylamine under the conditions used in the present work would be very small. It remains to be established whether the binding capacity of SSAO, acting as VAP-1, is modulated by the presence of substrate oxidation, during the inflammatory process. H_2O_2 formed during substrate oxidation is known to be involved in mediating some other functions of SSAO, including the promotion of glucose transport [see 1,8, 29]. In that case it has been proposed that methylamine, formed by the intracellular metabolism of adrenaline by monoamine oxidase, may be the physiological substrate [30].

The cell-surface acceptor for VAP-1 would be more complex than a free amino acid. The studies reported here show that some L-lysine derivatives also to interact with SSAO, although the simple substituted derivatives were considerably less potent as inhibitors. Steric factors may be important if interaction occurs at the active site, since substrates must penetrate a narrow entrance cavity in order to access the catalytic system [27,28]. Some small lysine-containing peptides have been reported to to inhibit SSAO [31,32]. Since the elastin is rich in lysine [19, 33], and many have reported on the role of SSAO in the maturation of the ECM [14, 34] we investigated the possible effects of soluble elastin on the activity of SSAO. The peptide was found to cause inhibition of the initial rates of oxidation of methylamine and benzylamine by the enzyme. The IC_{50} value was 4.6 mg/ml with benzylamine as substrate, which corresponded to approximately 66 μM. Fluorescence quenching assays showed that soluble elastin bound SSAO, in the presence of H_2O_2, with a K_d of approximately 0.1 mg/ml, a value that is nearly 50 times less than the corresponding IC_{50} value for inhibition of benzylamine oxidation. This contrasts with the behaviour of L-lysine, where the K_i value was similar to K_d. and would indicate that elastin also binds to sites that are different from the inhibitory binding site.

5. CONCLUSION

The binding *in vitro* of SSAO to lysyl residues might serve as a model of adhesion for SSAO/VAP-1. L-lysine and many of its derivatives are not oxidized by SSAO, but bind to the enzyme in the presence of the hydrogen peroxide produced as a result of the oxidation of benzylamine. Some lysine-containing peptides acting as inhibitors of SSAO have already been reported in some studies [35], [31], however the authors did not consider the effects of hydrogen peroxide, whose presence appears critical for the inhibition.

It is possible that the binding of SSAO/VAP-1 to its cell-surface bound target, may be regulated by the presence of a physiological substrate. This, after being oxidized by the enzyme, would produce the H_2O_2 necessary for the adhesion process. In a similar fashion, the interaction of SSAO with lysyl residues on the elastin precursor, tropoelastin, might be regulated by the production of hydrogen peroxide. The binding of SSAO to tropoelastin might partly explain the role of SSAO in the maturation of elastin in the ECM. However further studies are needed to confirm the validity of the model *in vivo* and to identify the target(s) and substrate(s) involved.

ACKNOWLEDGEMENTS

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11

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13

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Figure Captions

Scheme 1. Reaction of a specific enzyme inhibitor in the presence of a substrate. Two possible interaction modes for the inhibitor are shown. Binding to the free enzyme corresponds to a competitive effect, whereas binding to E.P (or E.S) represents an uncompetitive effect.

Fig.1. Effect of L-Lysine on the time courses of the SSAO-catalysed oxidation of benzylamine. (a) All samples contained SSAO (final protein concentration 0.010 mg/ml) in phosphate buffer (0.2 M, pH7.2). The reactions were started by the addition of benzylamine (5 mM) and their progress was monitored at 250 nm on a Cary 300-Bio spectrophotometer at 37 ^{O}C. Data points are representative of 4 different determinations and were fitted to first order exponential curves with the aid of GraphPad Prism, version 5.00. **(b)** Either D-lysine or L-lysine at the final concentration of 0.5 mM were added as inhibitors. When present, catalase was added at the final concentration of 0.15 units/ml. The reactions were started by the addition of benzylamine (5 mM) and their progress was monitored at 250 nm at 37 ^{O}C. All data points are the mean values from three different determinations. Error bars were omitted for clarity.

14

Fig. 2. The effects of preincubation with L-lysine in the presence or absence of hydrogen peroxide on SSAO activity. SSAO (0.010 mg/ml, final concentration) was preincubated, in a waterbath at 37 $^{\circ}$C, for different times with or without L-lysine (0.5 mM), in the presence or in the absence of 1 mM H_2O_2 and/or 0.15 units of catalase in 0.2 M phosphate buffer, at pH 7.4. The reactions were then started by the addition of 5 mM benzylamine and their initial rates were determined by direct detection of benzaldehyde at 250 nm. Values are the means $\pm$ S.E.M of 4 determinations; * corresponds to $p < 0.05$, ** to $p < 0.001$, *** to $p < 0.0001$ in comparison with the control (Ctrl) at the corresponding preincubation times.

Fig. 3. Determination of the kinetic parameters for the inhibition of SSAO by L-lysine. (a) Samples contained 0.1 mM hydrogen peroxide and increasing concentrations of L-lysine (from 0 to 1 mM) and benzylamine (from 0 to 2 mM). The values of K_m/V_{max}, obtained fitting the data to the Michaelis-Menten equation, were invariant to the increasing of the concentration of inhibitor used (as evident from the parallel double reciprocal plots shown in the inset). Data shown ($v = A_{250} / min$) are the mean values from four different experiments. **(b)** The time-courses data, such as those shown in Fig. 1, were fitted to equation (1) described in the text to determine the values of k'. The slope of the line obtained is then used to obtain an approximate K_i value (103 ± 14 μM) using equation (3).

Fig. 4. Determination of IC_{50} for the inhibition of SSAO by soluble elastin. The initial rates of formation of hydrogen peroxide, derived by the oxidation of benzylamine by SSAO, in the presence of soluble elastin at concentrations ranging from 0 to 10 mg/ml were determined by the spectrophotometric method by Holt et al (2006), described in the text. Reactions were followed at 37 $^{\circ}$C. The sigmoidal plot was obtained by fitting the response values, expressed as absorbance·min^{-1}·10^{-3} (v in the figure) against the logarithm of the concentration of inhibitor used. The curve fit and the value of IC_{50} of 4.60 ± 0.04 mg/ml were obtained with the aid of the computer software GraphPad Prism, version 5.00.

Fig. 5. Determination of the binding of L-lysine to SSAO. The fluorescence emission spectra maxima for SSAO, in the emission range 300-500 nm, at the excitation wavelength of 270 nm, were obtained in a Perkin Elmer Spectrofluorimeter upon stepwise titration of 3.33 nM SSAO with increasing concentrations of L-lysine, from a concentration of 10 µM up to a total concentration of 6 mM, in the presence and in the absence of 0.1 mM of H_2O_2 . When lysine was present in the absence of externally added H_2O_2, 10^{-3} U of catalase were added, to avoid interference from the variable amounts of H_2O_2 contained in some batches of L-Lysine. The relative fluorescence quenching (ΔF) values were corrected for background and plotted against the concentration of added L-lysine. Data were fitted to a hyperbolic binding curve with the aid of Graphpad Prism, version 5.0. All points shown above are the mean values plus S.E.M. of at least six different determinations, error bars were less than the representation of the points.

15

Fig. 6. Determination of the binding of soluble elastin to SSAO
Titration of 3.33 nM SSAO with increasing concentrations of soluble elastin, in the concentration range from 0 up to 0.25 mg /ml (approximately 3.54 µM) and in the presence of 0.1 mM H_2O_2, was performed as described in Fig. 6. All points shown above are the mean values plus S.E.M. of at least six different determinations, error bars were less than the representation of the points. The non-specific binding obtained in the presence of catalase was subtracted from the curve obtained in the presence of H_2O_2 resulting in a hyperbolic curve with a K_d of 0.05 ± 0.02 mg /ml, corresponding to approximately 0.70 µM.

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slope= [1 + (Km / S)] Ki / k

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