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Extended Substrate Specificity of Serum Amine Oxidase: Possible Involvement in Protein Posttranslational Modification

Xintao Wang,*,† Paola Pietrangeli,* Mircea-Alexandru Mateescu,† and Bruno Mondovi*,[1]

**Department of Biochemical Sciences "A. Rossi Fanelli" and CNR Center of Molecular Biology, Rome University "La Sapienza," P-le. Aldo Moro 5, 00185, Rome, Italy; and †Department of Chemistry, Université du Québec à Montréal, C. P. 8888, Succ. A., Montréal, Québec, H3C 3P8, Canada*

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The capacity of bovine serum amineoxidase (SAO) to oxidize free amino groups of nonconventional substrates, such as polylysine (up to 50 kDa) and some proteins as lysozyme and ribonuclease A, is described. The oxidation was quantified from the amount of H_2O_2 and NH_3 enzymatically produced by SAO. Kinetic analysis indicated a stereospecific preference for L-configuration. Maximal oxidation rate was obtained with poly-L-lysine (9.6 kDa). After 10 h of incubation at 37°C, the poly-L-lysine was partially oxidized generating 1.5 moles of H_2O_2 by one mole of polylysine. Denatured SAO presented very low oxidation rates with the mentioned substrates.

Bovine serum amine oxidase (SAO) is a copper enzyme (EC 1.4.3.6) involved in catalysis of oxidative deamination of primary biogenic amines (spermine, spermidine, etc), following the reaction (1):

$$\text{R-NH}_2 + \text{H}_2\text{O} \xrightarrow{\text{SAO}} \text{R-CHO} + \text{H}_2\text{O}_2 + \text{NH}_3$$

There is a growing interest for this enzyme and important data have been accumulated concerning its structural and kinetic properties (2,3), inhibitors (4,5) and possible use as an antitumoral agent (6). During its purification on AH-Sepharose 4B column (7), a particularly high retention of the enzyme (sometimes irreversible) was observed in several cases (Mateescu et al., unpublished data), probably related to some modifications of separation conditions (running temperature, resin manufacturing, etc). We have hypothesized that, in these particular conditions, SAO is able to recognize the aminohexyl (AH) spacer as an affinity ligand and possibly as a modified substrate (in a certain extent similar to the alkylamino-terminal groups of spermine and spermidine SAO natural substrates).

Oda et al. (8) have found that ε-amino groups of some small synthetic lysyl peptides (2 to 6 amino acid residues) were oxidized by SAO with oxidation rates depending on the amino acid sequences. They have discussed the hypothesis that SAO, beyond its important role in regulation of the biogenic amines metabolism (9), can be involved in intermolecular cross-linkages between peptidic chains of elastin or collagen in aorta. It is known that elastin is oxidized by lysyl-oxidase (10) which is a copper-enzyme, but not a Tri-hydroxyphenylalanine quinone (TPQ) dependent enzyme (11). Recently, an indirect involvement of diamine oxidase (DAO) in protein modification by oxidized putrescine spontaneous incorporation into proteins was reported (12). So far, modification of soluble proteins by their direct oxidation catalyzed by TPQ depending amineoxidases, was not described.

Based on these data and on recent observation of a strong chromatographic interaction of SAO with Polylysine-Agarose (Mateescu et al., unpublished results), we have carried out this comparative study on the SAO catalyzed oxidation of (poly)lysine with different molecular weight and of

[1] To whom correspondence should be addressed. Fax: 39-6-4440062.

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some proteins, which is the first, at our knowledge, to show that: a) the SAO substrate specificity is not strictly limited to biogenic primary amines and small peptides and b) to reveal the ability of SAO to oxidize ϵ-amino groups of polypeptides and proteins. The stereospecificity and chain length influences on the reaction rate are also described.

MATERIALS AND METHODS

Reagents. Poly-D-lysine and Poly-L-lysine (MW 10–50 kDa), D-lysine, L-lysine, ribonuclease A, lysozyme, bovine serum albumin (BSA), bovine hemoglobin, α-amylase (Sigma, USA), horse-radish peroxidase (230 EU/mg) and Ammonia-Test-Combination (Boëhringer Mannheim, Germany) and the other chemicals (reagent grade), were used without further purification. The sodium salt of N,N-diethyldithiocarbamate (DDC) from Merck (Darmstadt) was recrystallized from ethanol.

Amine oxidases purification. Purified electrophoretically homogeneous SAO was obtained as recently described (13), with a specific activity of 0.26–0.33 EU/mg protein. The SAO activity was assayed spectrophotometrically with benzylamine as substrate (Tabor et al. [14]).

Determination of H_2O_2 generated from (poly)peptide substrates as indicator of SAO catalyzed oxidation. a) Fast qualitative procedure: the release of H_2O_2 under SAO catalysis was evidenced by a fast peroxidase/benzidine test adapted from Brad et al. (15) method. Volumes of 0.2 mL SAO (4 mg/mL) were added to test tubes containing 1 mL of poly-L-lysine or poly-D-lysine (20 mg/mL) and incubated (0, 2, 4, 6 and 24 hours) for the enzyme reaction (sol. A). After each incubation time, 0.2 mL of solution A were added to test tubes, each one containing 0.2 mL benzidine (25%) reagent (sol. B). H_2O_2 was detected by adding 0.05 mL peroxidase (0.5 mg/mL). The fast appearance of a blue colour (characteristic of oxidized benzidine) was a proof of the H_2O_2 release by SAO. As negative controls, BSA, bovine hemoglobin and heat denatured SAO (95°C for 4 min, exhibiting no more enzymatic activity) were tested instead of native SAO.

b) Quantitative determination of H_2O_2 was accomplished by the fluorimetric method of Makatoshi et al. (16), based on the conversion rate of homovanillic acid (HVA) into a highly fluorescent compound by the released H_2O_2, under peroxidase catalysis. The fluorimetric standard curve was prepared by first incubating for 10 min a reaction mixture of 0.1 mL of 30 mM potassium phosphate buffer (pH 7.2), 0.1 mL horse-radish peroxidase (0.5 mg/mL), 0.1 mL homovanillic acid (1 mg/mL), 0.1 mL polylysine (10 μM) and 0.1 mL of H_2O_2 with serial concentrations from 0 to 20 μM; then 2 mL of 0.1 N NaOH was added to the mixture. The fluorescence was excited at 324 nm and read at 426 nm on a "Jobin Yvon Spectrofluo" /JY 3D/.

The effect of polylysine (9.6 kDa) on SAO enzymatic activity measured with benzylamine (14), was also studied. The concentration of polylysine was equal to that of benzylamine in terms of amino groups concentration.

The influence of (poly)lysine substrate concentration on the SAO enzymatic oxidation rate. The initial oxidation rate of (poly)lysine, incubated for one hour with SAO (0.04 mg/mL reaction medium), was established as a function of (poly)lysine concentration. The Michaelis constant (Km) and maximal velocity (V_{max}) values for lysine and polylysine substrates were obtained by the double reciprocal plot method.

Evaluation of the oxidation extent of polylysine at optimized concentration catalyzed by SAO. The reaction time course under SAO (final conc.: 0.04 mg/mL) catalysis was monitored at 37°C. The H_2O_2 was determined by the fluorimetric method (16). The reaction mixtures containing 0.1 mL of 20 mM phosphate buffer (pH 7.4), 0.1 mL SAO (0.2 mg/ml) and 0.1 mL (poly)lysine substrate (0.5 mg/mL) were incubated at 37°C for various intervals in the range 0–10 h; after each interval 0.1 mL HVA (1 mg/mL) and 0.1 mL peroxidase (0.5 mg/mL) were added and incubated again for 30 min. The reaction was stopped with 2 mL of 0.1 N NaOH and the amount of H_2O_2 produced was determined from the standard curve.

Evaluation of the SAO ability to oxidize (poly)lysine of different molecular weights and configuration. Poly-D-lysine and poly-L-lysine of two molecular weights (9.6 and 50 kDa), as well as D-lysine and L-lysine, were tested as SAO substrates, all at the same concentration of 0.1 mg/mL. The oxidation rate was determined after one hour of incubation at 37°C. The maximal oxidation rate was taken as 100%; the other oxidation rates were expressed as the percentage from the maximal rate.

Modification of some native proteins by SAO. The production of H_2O_2 and NH_3 was considered as indicator for the oxidative deamination of the free amino groups of the proteins tested as possible substrates for SAO: BSA (67 kDa), bacterial (*Bacillus subtilis*) α-amylase (50 kDa), lysozyme (14.6 kDa) and ribonuclease A (13.7 kDa). Each protein (20 mg/mL) was incubated with SAO (final concentration: 0.04 mg/mL) and the released H_2O_2 was determined as for polylysine. The full scale (100) of the fluorimeter was calibrated with 2 nmoles of H_2O_2 in the presence of the protein to be tested. Controls have been carried out with two forms of inactivated SAO: a) heat-denatured SAO (95°C for 4 min) which exhibited no enzyme activity; b) SAO treated with excess chelating agent (20 mM DDC) with no activity (prepared as Morpurgo et al. [17]). Separate assays were done with heat denatured substrates. The proteins (ribonuclease A and lysozyme) were heated at 95°C for 4 min, prior to be used as substrate. The ribonuclease A with free amino group blocked by citraconylation (treatment with citraconic anhydride [18]) was also tested as a substrate. The generation of NH_3 was spectrophotometrically determined following an enzymatic method of Bergmeyer and Beutler (19).

RESULTS AND DISCUSSION

Both poly-L-lysine and poly-D-lysine can be recognized as substrates and oxidized by SAO with production of H_2O_2. This oxidation should be specifically ascribed to native SAO, because no H_2O_2 was formed with heat denatured SAO or with other proteins (BSA and hemoglobin).

The oxidation rate of (poly)lysine is relatively low compared with that of benzylamine substrate. Unlike other substrates which can be oxidized by SAO in 0.1 M potassium phosphate buffer (pH 7.2), in case of polylysine a strong inhibitory effect on the enzymatic oxidation was produced by this medium: even after 24 h of incubation, no H_2O_2 could be detected with either poly-L-lysine or poly-D-lysine as substrate. This aspect fits well with Stevanato et al. (20) reports describing an inhibitory effect on SAO activity imputable to high ionic strength. Therefore our experiments were carried out in 0.01 M K-phosphate buffer, pH 7.2. We have also found that polylysine did not significantly affect the SAO oxidation kinetics of benzylamine (data not shown).

The oxidation rate of polylysine varied with the D or L configuration and with the molecular weight (Fig. 1). The highest oxidation rate (1.4 moles H_2O_2/mole polylysine-h) generated with poly-L-lysine (9.6 kDa) was taken as 100%. At the same substrate concentration (0.1 mg/mL), the oxidation rates of lysine monomers (both configurations) were lower than those of the corresponding polymer of 9.6 kDa and comparable with those of the polymer of 50 kDa (except the D-configuration, for which poly-D-lysine exhibits the lowest oxidation rate). For substrates with the same steric configuration, the oxidation rate is maximal for 9.6 kDa and then decreases with the increase of molecular weight. For polylysine, with the same increase of molecular weight from 9.6 kDa to 50 kDa, the oxidation rate decreased by 58% in case of poly-L-lysine and by 63% for poly-D-lysine.

For polylysine chains of same molecular weight, SAO exhibited a steric preference for the L configuration (Fig. 1). The oxidation rate of poly-D-lysine (9.6 kDa) was 57% from that of poly-L-lysine (9.6 kDa). For poly-D-lysine (50 kDa) the rate was only 50% from that of poly-L-lysine (50 kDa). The SAO sterical preference for the L configuration of non-classical substrates appears normal, showing that the enzyme is adapted for a better transformation of L-polypeptides (based on L-aminoacids) characteristic for natural systems, than of D-polypeptide series. These results fit well with data showing SAO stereospecificity in oxidation of dopamine (21) and of stereospecifically deuterated benzylamines (22).

The specificity constant (k_{cat}/K_m) gives an image of overall enzyme catalytic efficiency. For the same configuration of polylysine, SAO shows preference for the lower molecular weight. For

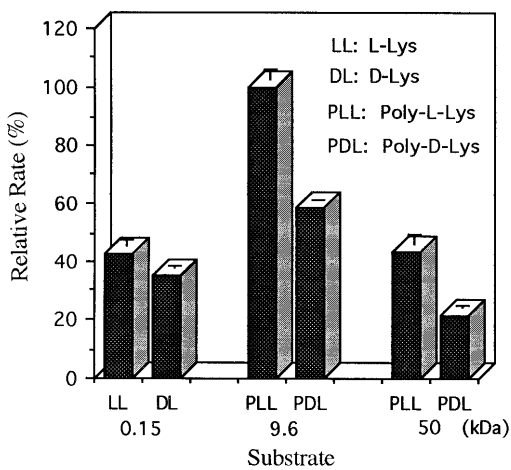

FIG. 1. Relative oxidation rates for different (poly)lysine substrates: 1 h of incubation with SAO (final concentration of 0.04 mg/mL) at 37°C; Lys stands for lysine.

instance, the specificity constant for poly-L-lysine (9.6 kDa) was 2.2 $M^{-1}s^{-1}$ while for poly-L-lysine (50 kDa) the value was of 0.14 $M^{-1}s^{-1}$. For the same molecular weight (e.g. 50 kDa), poly-L-lysine (k_{cat}/K_m 1.4×10^{-1} $M^{-1}s^{-1}$) is a better substrate than poly-D-lysine (k_{cat}/K_m 6.9×10^{-2} $M^{-1}s^{-1}$), clearly showing a sterical preference for the L-form. Both configurations of polylysine are definitely better substrates than L-lysine ($k_{cat}/K_m 4.4 \times 10^{-5}$ $M^{-1}s^{-1}$) or D-lysine (k_{cat}/K_m 5.4×10^{-5} $M^{-1}s^{-1}$), showing the enzyme preference for polymeric substrate. These data fit well with the chromatographic retention of SAO on Polylysine-Agarose (Mateescu et al. unpublished data). Since best oxidation rates were obtained with poly-L-lysine, the rest of kinetic analysis were done with this substrate.

The influence of polylysine concentration on the oxidation rate is of Michaelis type (Fig. 2). The oxidation rates increased with the increase of poly-L-lysine concentrations up to 0.4 mg/mL for both molecular weights (9.6 kDa and 50 kDa). With poly-L-lysine (50 kDa) the oxidation rate was stabilized at the saturating concentration, while with poly-L-lysine (9.6 kDa) a slight substrate-inhibitory effect was observed at higher concentration (Fig. 2). Therefore a polylysine substrate concentration of 0.1 mg/ml was used for all successive experiments.

The oxidation extent of polylysine has been evaluated from the oxidation reaction time course (Fig. 3). The SAO concentration was 0.04 mg/mL (within the range of physiological concentration [23]). The amount of H_2O_2 released with poly-L-lysine (9.6 kDa) was of about five times higher (Fig. 3) than the amount generated with poly-L-lysine (50 kDa). When the oxidation extent was expressed in terms of moles H_2O_2/mole polylysine, the final oxidation extent for both poly-L-lysine (9.6 kDa and 50 kDa) was about the same: 1.5 moles H_2O_2/mole polylysine. This fact suggests that only a limited number of amino groups per molecule of polylysine, probably in terminal positions, are oxidized by SAO.

As far as the possible involvement of SAO in protein modification is concerned, it was observed that not all proteins of different size tested as possible substrates could be oxidized by SAO. Only the proteins with a relative low molecular weight as lysozyme (14.6 kDa) and ribonuclease A (13.7 kDa), were readily oxidized by SAO with the production of H_2O_2 and NH_3. The other proteins with larger size (BSA: 67 kDa, bacterial α-amylase: 50 kDa) were not oxidized by SAO. This aspect fits well with the results of the influence of polylysine molecular weight on the oxidation rate: best substrate was the peptide of 9.6 kDa, while the larger polymer (50 kDa) was clearly a poor substrate (Fig. 1). Relatively small polylysine and proteins probably fit better with the size of hydrophobic pocket (24) of the SAO active site. The ionized amine groups of lysyl residues (probably interacting

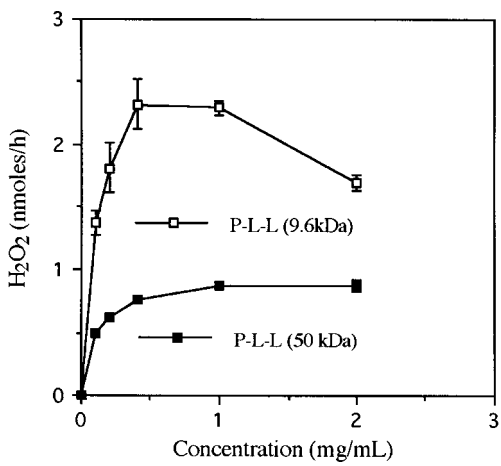

FIG. 2. Influence of poly-L-lysine (PLL 9.6 kDa and 50 kDa) substrate concentration on the oxidation rate (expressed as the total amount of H_2O_2 generated after 1 h of incubation at 37°C). The concentration of SAO was of 0.04 mg/mL.

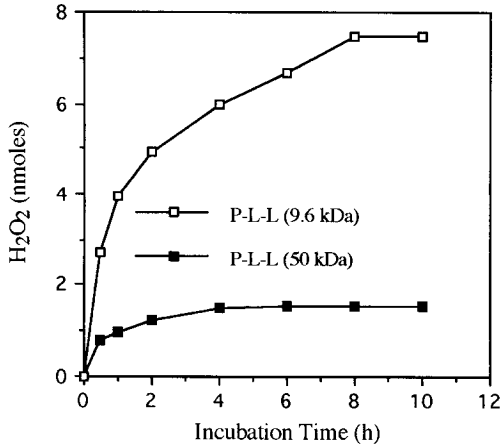

FIG. 3. Time course of the poly-L-lysine (0.1 mg/mL) oxidation by SAO (0.04 mg/mL). The oxidation extent was expressed as the amount of H_2O_2 enzymatically generated as a function of incubation time, at 37°C.

with the anionic centers of the SAO active site [20]) and the sterical configuration (preference for L- series) are also favorable factors for the polypeptide oxidation as substrates. Since both lysozyme and ribonuclease have adequate size and enough lysyl residues (6/129 for lysozyme [25] and 10/124 for ribonuclease A [26]), they can relatively easy be recognized and oxidized by SAO. It is worth to mention that among the proteins tested, only the lysosyme and the ribonuclease A were found as possible substrate for SAO and only these two proteins have lysine as N-terminal residue. Is this another factor explaining the SAO preference in their recognition and oxidation? This aspect will be dealt with in another work.

The fact that L- and D-lysine monomers are poor substrates (based on specificity constant) is probably related to the different distribution of charges (due to a free carboxyl) which limits its recognition by the SAO active site anionic center.

In order to evaluate the role of molecular and conformational integrity for the polypeptide enzymatic oxidation, a double-approach investigation was carried out by altering the structures of both substrate (ribonuclease A) and enzyme (SAO). The results are shown in Fig. 4. In order to exclude the possibility of undesirable contamination of ribonuclease A (13,6 kDa) with biogenic amines or low MW polypeptides, experiments were carried out with ribonuclease preparations submitted to dialysis (tubes retaining more than 90% compounds with more than 12,4 kDa after 15 hrs of dialysis). The oxidation rate of the native substrate with the native enzyme (0.1 nmole H_2O_2/h) was taken as 100%. With heat-denatured substrate (ribonuclease A) an increase (by 26%) of the oxidation rate was observed. Probably, its secondary structure is partially altered by heating, making more free amino groups accessible to the SAO catalytic center. No H_2O_2 was produced by SAO with blocked ribonuclease A (75% citraconylation). Heat-denatured SAO (with no benzylamine activity) exhibited no activity for the oxidation of the protein substrate. Copper-chelated SAO presented a negligible protein oxidation rate (less than 2% of the optimal rate with the native SAO and native substrate [Fig. 4]).

As a confirmation of the oxidative deamination, NH_3 (the other product of the SAO reaction), was also found in amounts comparable with those of H_2O_2. Furthermore, the H_2O_2 production was also confirmed by a recent method (27) based on 3-amino-1,2,4-triazole dependent irreversible inhibition of catalase. With citraconylated ribonuclease A (free amino groups blocked), no NH_3 could be detected when incubated with native SAO. With heat-denatured SAO no NH_3 was detected. Therefore, it appears that the ability of SAO to oxidize polypeptides and proteins as substrate, depends not only on the existence of free amino groups but also on the molecular weight,

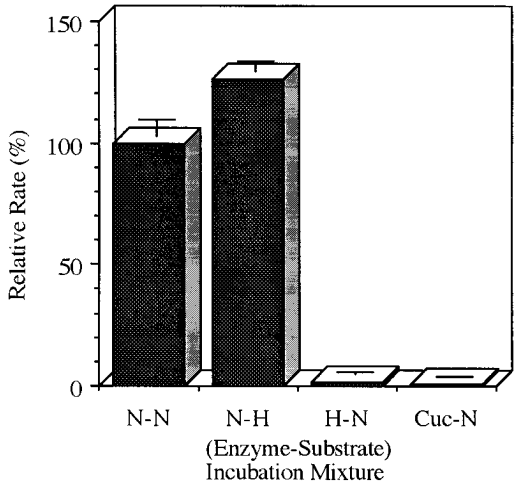

FIG. 4. Relative oxidation rates with native and different denatured forms of enzyme (SAO) and substrate (ribonuclease A). N-N refers to native enzyme with native substrate; N-H: native enzyme with heat-denatured substrate. H-N: heat-denatured enzyme with native substrate. Cuc-N: Copper chelated enzyme with native substrate.

on the steric configuration and on the overall electrostatic environment of the amino groups of the substrate. At the same time the molecular and conformational integrity of the active form of the SAO enzyme is essential for the substrate oxidation.

In conclusion, the results here presented show that SAO, has a larger substrate specificity and beyond its main function as modulator of biogenic amine concentration, can play a role in the process of protein post-translational modification, like the lysyl oxidase (10). Further investigation needs to be carried out to elucidate the mechanisms of the involvement of SAO in protein modification.

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