Heterologous Expression and Characterization of Mouse Spermine Oxidase*

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Polyamine oxidases are key enzymes responsible of the polyamine interconversion metabolism in animal cells. Recently, a novel enzyme belonging to this class of enzymes has been characterized for its capability to oxidize preferentially spermine and designated as spermine oxidase. This is a flavin adenine dinucleotide-containing enzyme, and it has been expressed both in vitro and in vivo systems. The primary structure of mouse spermine oxidase (mSMO) was deduced from a cDNA clone (Image Clone 264769) recovered by a database search utilizing the human counterpart of polyamine oxidase. This is a flavin adenine dinucleotide-containing enzyme, and it has been expressed both in vitro and in vivo systems. The primary structure of mouse spermine oxidase (mSMO) was deduced from a cDNA clone (Image Clone 264769) recovered by a database search utilizing the human counterpart of polyamine oxidase.

Polyamine oxidase (PAO),1 a flavin adenine dinucleotide (FAD)-containing enzyme, catalyzes the oxidation of polyamines at the secondary amino group, giving different products according to the organism considered. In particular, vertebrate PAOs (EC 1.5.3.11) participate in the interconversion metabolism of polyamines, converting N-acetyl derivatives of spermine (N-acetylSpm) and spermidine (N-acetylSpd) into Spd and putrescine, respectively, plus 3-aminopropanal and H2O2 (1–3). PAOs with similar characteristics occur in methylotrophic yeasts (4, 5) and amoebae (6). On the contrary, plant (1), bacterial (7), and protozoan (8) PAOs oxidize spermidine and spermine to 4-aminobutanol or N-(3-aminopropyl)-4-aminobutanol, respectively, plus 1,3-diaminopropane and H2O2. As these compounds cannot be converted directly to other polyamines, this class of PAOs generally is considered to be involved in the terminal catabolism of polyamines. Since PAOs play a crucial role in polyamine catabolism, these enzymes are important drug targets, and in fact, it has been shown that a number of polyamine analogues have an antitumor effect in different cell lines (9–12).

As compared with large and detailed investigations on plant PAOs (1, 13–22), only little attention has been devoted to the animal counterpart (2, 23–28). Recently, Wang et al. (29) and Vujic et al. (30) have reported the cloning and characterization of novel mammalian PAO enzymes capable of oxidizing preferentially Spm and for this reason named spermine oxidase (SMO) (30). In particular, this enzyme was expressed in an in vitro transcription/translation system (29) and into transiently transfected human kidney cells (30). Based on these studies, it was postulated that in addition to the traditional interconversion pathway in which Spm is first acetylated by spermidine/spermine N-acetyltransferase and then oxidized by PAO, mammalian cells contain an enzyme capable of directly oxidizing Spm to Spd (30).

Data base searching analysis using PAO1 cDNA sequence recovered a mouse cDNA clone (Image Clone 264769) corresponding to the murine counterpart, which was supplied by the United Kingdom Medical Research Council Human Genome Mapping Resource Centre (Cambridge, United Kingdom) consortium and herein defined as mSMO. To enhance the knowledge of enzymology of mammalian SMO and shed light on the structure/function relationship of this enzyme, the mSMO cDNA was further subcloned and expressed in secreted and secreted-tagged forms into Escherichia coli BL21 DE3 cells. This paper describes the expression and the main biochemical features of mouse spermine oxidase. Notwithstanding the low amino acid sequence homology shown between MPAO and mSMO, molecular modeling of mSMO based on MPAO three-dimensional structure (18) suggests that the general features of MPAO active site are conserved in mSMO.

EXPERIMENTAL PROCEDURES

Chemicals—N,N′-bis(2,3-butadienyl)-1,4-butanediim (MDI) was a generous gift from Dr. M. De Agasio (Consiglio Nazionale delle Ricerche, Area della Ricerca di Roma, Montelibretti, Italy). Spm, Spd, and pargyline were purchased from Sigma. N′-acetylSpm and N′-acetylSpd originally purchased from Sigma are no longer commercially available.

DNA and Genetic Methodology—The methods described by Sambrook et al. (32) were used for the extraction and manipulation of plasmid DNA and general DNA in vitro methods. Nucleotide sequenc-
ing was obtained for both strands using the automated fluorescent dye terminator technique (PerkinElmer ABI model 373A). Analyses of data base sequences used on-line facilities of the NCBI (www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using the program ClustalW (available at www.ebi.ac.uk/clustalw) (33).

Construction of mSMO Expression Plasmids—A BLAST sequence similarity search using the human cDNA PAO1 (GenBank accession number AY039889) resulted in the Image Clone 2647695 (GenBank accession number BC004831), which was supplied by the United Kingdom Medical Research Council Human Genome Mapping Resource Centre. To test the fidelity of this sequence, the murine cDNA was resequenced, and by PCR amplification, two full-length cDNAs were generated possessing modified 5' and 3' ends. In particular, the two following oligonucleotides were used to introduce the XhoI restriction site and produce a stop codon and, alternatively, a longer open reading frame in-frame with the downstream His-tagged sequence at the 3' end of mSMO cDNA: PAO1-REV, 5'-AAATATCTCGAGGGAAACATTTGGCAGTGAGG-3', and PAO2-REV, 5'-TTATACTCGAAGGGCCTCTGTCGAAGGGTC-3', respectively. The oligonucleotide PAO3-FOR, 5'-CCATGCAAAGTTGTGAATCCAG-3', was used coupled with the above described primers. Amplified PCR products were restricted by XhoI and ligated with the restricted pET25b vector (Novagen) to obtain two genetic constructs. The first of these named pmSMO has a bacterial periplasmic leader sequence at the 5' end. The second one named pmSMO-HT has a bacterial periplasmic leader sequence and a His tag sequence at 3' end. These two recombinant cDNA constructs were utilized to transform E. coli BL21 DE3 competent cells. Expression of mSMO in E. coli cells—E. coli BL21 DE3 cells transformed with pmSMO and pmSMO-HT plasmids were grown at 30°C in LB medium containing 50 μg/ml ampicillin to Aopt = 0.6 and then induced with isopropyl-β-D-thiogalactopyranoside (1 mM final concentration) followed by further cultivation for 5 h at 28°C.

Periplasmic Fraction Purification—The E. coli BL21 DE3 cells were harvested by centrifugation at 4°C for 10 min at 10,000 × g, washed with 0.4 culture volumes of 30 mM Tris-HCl, pH 8.0, 20% sucrose, and 1 mM EDTA, and incubated 5–10 min at room temperature. The suspension was centrifuged at 10,000 × g for 10 min at 4°C. The pellet was resuspended in 0.05 culture volumes of ice-cold 5 mM MgSO4 with vigorous shaking for 10 min on ice. The cell was then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant corresponding to the periplasmic fraction was finally collected.

Rapid Affinity Purification with pET His Tag Systems—The supernatant from E. coli BL21 DE3 cells transformed with the plasmid pmSMO-HT was applied to a column (3 ml) with Ni2+ cations immobilized on the His-Bind resin (Novagen) equilibrated with binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). The column was washed with 60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, followed by elution with 750 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9.

Assay of mSMO and Determination of the Kinetic Constants—Enzyme activity was measured spectrophotometrically by following the formation of a pink adduct (ε155 = 2.6 × 10³ M⁻¹ cm⁻¹) as a result of the oxidation and following condensation of aminotyrosine and 3,5-dichloro-2-hydroxybenzenesulfonic acid catalyzed by horseradish peroxidase (14). The measurements were performed in 0.2 mM sodium phosphate (NaPi) buffer, pH 8.0, with different substrates at various concentration. Enzyme activities were expressed in international units (IU, the enzyme amount catalyzing the oxidation of 1 μmol of substrate × min⁻¹) on 1 liter of culture broth. Protein content was estimated by the method of Bradford (34) with bovine albumin as a standard. SDS-PAGE was performed according to the method of Laemmli (35). In the enzyme assays, the mSMO concentration was 2.0 × 10⁻⁸ M. Kₘ and kcat values were determined with Spm as a substrate at pH 8.0. In the mSMO inhibition assays, the mononuclear oxidase inhibitor pargyline and the PAO inhibitor MDL72527 were used at the final concentration of 1.0 mM with Spd concentration of 0.5 mM. The concentrations chosen for each inhibitor were based on studies published previously (2).

Identification of mSMO Reaction Products—The enzymatic reaction was carried out in a final volume of 1 ml containing 0.1 mM NaPi, pH 8.0, 0.2 mM Spm, and 0.1 unit of purified mSMO. Incubation was performed at 37°C for 10 min and stopped by the addition of 200 μl, 20% (w/v) HClO₄. Precipitated proteins were removed by centrifugation, and the supernatants were dansylated and analyzed by silica gel TLC according to Flores and Galston (36) or oxidized by maize MPAO enzyme. The

![Fig. 1. Purified recombinant mSMO-HisTag protein analysis. SDS-PAGE analysis of the recombinant mSMO-HisTag protein (1 μg of the purified enzyme) after Coomassie Brilliant Blue staining. MW, molecular weight marker (low range, Sigma).](image1)

![Fig. 2. Amine oxidation reaction catalyzed by mSMO. Scheme of the cleavage on the Spm substrate operated by mSMO.](image2)

![Fig. 3. Absorbance spectra of mSMO: effect of adding Spm to oxidized enzyme and oxygenation of the reduced form. Absorbance spectra were recorded in anaerobiosis with 1 ml of 10 μM mSMO in 0.1 M NaPi, pH 7.5, using a Thunberg-type cuvette. a, native enzyme in anaerobic condition; b, native enzyme in anaerobic condition + 20 μM Spm; c, enzyme after reoxygenation.](image3)
amounts of O2 consumed and H2O2 formed from Spm by the oxidation with mSMO and from oxidized Spm by the oxidation with MPAO were measured as described in Federico et al. (37).

Molecular Modeling of mSMO—The molecular model of mSMO was built using the crystal structure of MPAO as a template (Protein Data Bank code 1B37) (18). In detail, a multiple sequence alignment among mSMO, MPAO, and other PAOs with known amino acid sequence was obtained using the program ClustalW (33). The alignment was then manually refined on the basis of mSMO secondary structure prediction obtained using the Predict Protein server (38) available online (dodo.cpmc.columbia.edu/pp/predictprotein.html), which checks for the occurrence of insertions and deletions on surface loops. Based on this alignment, the three-dimensional structure of mSMO was then built using Modeler (Release 6), a program that models protein three-dimensional structure by satisfaction of spatial restraints (39).

RESULTS

Expression of mSMO in E. coli Cells—The mSMO cDNA clone was obtained as described under "Experimental Procedures." The two recombinant cDNA constructs pmSMO (secreted form) and pmSMO-HT (secreted-tagged form) were used to transform E. coli BL21 DE3 cells. After induction with isopropyl-β-D-thiogalactopyranoside under the control of the T7 promoter, the catalytically active proteins were both expressed at

Fig. 4. Amino acid sequence comparison of animal (mSMO and PAOh1) and plant (MPAO1, BPAO1, BPAO2, and APAO) polyamine oxidase proteins. Multi-alignment was done using the program ClustalW sequence alignment. Identical residues are indicated by gray boxes. Signal peptides are underlined. Residues in the maize MPAO enzyme organizing the catalytic U-tunnel are in boldface letters: the ones putatively involved in the catalytic activity are labeled by an asterisk, the ones composing the tunnel entrance (carboxylate ring and aromatic portion) are labeled by a ¥. Numeration is shown at the right side. Percentage of identity refers to mouse SMO (Image Clone 264769). BPAO1 and BPAO2, barley PAOs; APAO, Arabidopsis thaliana PAO.
Fig. 5. Stereoview of the residues building up the catalytic tunnel of MPAO (A) and mSMO (B). The isoalloxazine ring of FAD is shown in green, whereas the substrate spermine is shown in red. Enzyme substrate complexes have been modeled using as a template the structure of MPAO in complex with the Spm analogue MDL72527 (Protein Data Bank code 1BQ5) (18). Residues are numbered according to MPAO amino acid sequence. The figure has been made with Grasp (41).

Table I

| Function in polyamine oxidase | MPAO (aa Pos.) | hMAO A (aa Pos.) | hMAO B (aa Pos.) | mSMO (aa Pos.) |
|------------------------------|----------------|------------------|------------------|---------------|
| Mainchain N and side-chain OH compensate for pyrophosphate negative charge | Ser 15 - 21 - 22 Alad 85 | Glu 35 - 40 - 41 - 55 | Ala 36 - 41 - 42 - 56 | Arg 43 - 48 - 49 - 63 |
| Compensates for pyrophosphate-negative charge | Arg 43 - 48 - 49 - 63 | Gly 57 - 64 - 65 - 77 | | |
| Carbonyl oxygen bridged by water molecule to N5 of isoalloxazine | Val 237 - 231 - 232 Gly 284 | Asn 59 - 66 - 67 - 79 | | |
| Main-chain NH makes H-bond with pyrimidine | Trp 60 - 67 - 68 - 80 | Ser 403 - 405 - 296 - 367 | | |
| Amino group bridged by water molecule to N5 of isoalloxazine | Lys 300 - 305 - 296 - 367 | Tyr 399 - 403 - 394 - 478 | | |
| Main-chain atoms H-bond with pyrimidine | Trp 393 - 397 - 388 - 472 | Thr 402 - 406 - 397 - 481 | | |
| Main-chain N compensates for pyrophosphate negative charge | Glu 430 - 435 - 426 - 519 | Tyr 439 - 444 - 435 - 528 | | |
| Extensive side-chain Van der Waals interactions with flavin dimethylbenzene ring | Val 440 - 445 - 436 - 529 | | | |
| Part of Phe-403 sandwich; Side-chain oxygen close to inhibitor carbons | | | | |
| Main-chain NH H-bonds with carbonyl at C2 of pyrimidine | | | | |

a level of about 6 units/liter of culture broth. The enzyme activity for both recombinant forms was measured spectrophotometrically as described under “Experimental Procedures.”

Protein Purification of mSMO—mSMO was isolated from E. coli BL21 DE3 cells by overexpression of pmSMO-HT and purified by using His-Bind chromatography kit (Novagen). The SDS-PAGE electrophoretic analysis was performed on pmSMO-HT transformed E. coli extract, and mSMO was purified. The last one results in a single band of ~68 kDa as shown in Fig. 1.

Kinetic Properties of mSMO—The substrate specificity of mSMO for several kinds of polyamines and acetylpolyamines has been investigated under standard conditions at pH 8.0. The purified enzyme could oxidize Spd rapidly and Spd extremely slowly (N2-acetyladaverine and N2-acetylpolyamines). The oxidation rate for N2-acetyladaverine was approximately 1:1,000 of that for Spm, whereas the rates for Spd and N2-acetylpolyamines were >3,000-fold lower than that for Spm, indicating that Spm is the preferential substrate for mSMO. No activity was detected using putrescine and N2-acetyladaverine as substrates.

The TLC analysis of reaction products (data not shown) demonstrated that Spm was oxidized by mSMO to Spd. Stoichiometric analysis of Spm oxidation by mSMO and Spm oxidation by MPAO yielded a molar ratio of substrate to O2 and H2O2 of 1:1. These results confirmed that mSMO converts Spm into Spd plus H2O2 and 3-aminopropanol (Fig. 2). The purified mSMO exhibited a pH optimum of 8.0 in NaPi buffer; thus, the kinetic properties of the secreted-tagged recombinant enzyme were determined using Spm as substrate at pH 8.0. The values of Km and kcat resulted to be 90 µM and 4.5 s-1, respectively. The absorption spectrum of the native enzyme showed three peaks typical of oxidized flavoproteins (15) with maxima at 278, 365 and 450 nm. The addition of equimolar amounts of substrate (Spm) in anaerobic conditions induced the reduction of the enzyme as indicated by the decrease of the absorbance bands in the visible range at 365 and 450 nm, whereas reoxygenation of the enzyme restored the initial spectrum, confirming the involvement of the flavin cofactor in the catalytic cycle (Fig. 3). The determination of the flavin content in the protein results in a flavin/mSMO stoichiometry of 1:1. To confirm that the enzyme activity was attributable to a PAO and not to monoamine oxidases, their specific inhibitors,
MDL72527 and pargyline, respectively, were included in the reactions. Only MDL72527 inhibited mSMO. The inhibition was complete in our experimental conditions. Substrate specificity and the pH optimum were also determined for the secreted recombinant form, measuring the enzyme activity of the periplasmic fraction, thus confirming the data obtained for the purified form.

**Structural Analysis and Active Site Modeling of mSMO—**

The primary structure of mSMO was deduced from the cDNA sequence. Its open reading frame predicts a 555-amino acid protein with a calculated Mr of 61,8523. Amino acid sequence alignment between mSMO and PAOh1 (29) has revealed that they share a 95.1% sequence identity, whereas the sequence comparison with the plant PAOs shows a lower identity ranging from 23.4 to 26.5%. Multiple amino acid sequence alignments of these two proteins together with other known members of the plant PAO family are shown in Fig. 4.

Given the fairly low sequence identity between mSMO and MPAO (26.5%), homology modeling techniques, which heavily rely on the availability of a correct sequence alignment, must be applied carefully to allow the construction of a reliable three-dimensional model of the entire mouse protein structure. For this reason, a multiple sequence alignment among mSMO, MPAO, and other PAOs with known amino acid sequence was obtained using the program ClustalW (Fig. 4) (33). In addition, the alignment was manually refined on the basis of mSMO secondary structure prediction, which was obtained using the program Predict Protein (38), to avoid the unlikely occurrence of insertions and deletions within secondary structure elements. This procedure yielded the final alignment between mSMO and MPAO, the only enzyme belonging to this class with known three-dimensional structure (18). Based on this alignment, the three-dimensional model of mSMO was built with the program Modeller (39).

As shown in Table I, an analysis of mSMO FAD binding pocket reveals a high degree of conservation of the residues involved in the stabilization of this prosthetic group among PAO and human monooamine oxidase enzymes (31). This result is not surprising, although it can be used as an internal check for the correctness of the sequence alignment used for mSMO model construction.

Fig. 5 shows a comparison of the active site structure of MPAO and mSMO. In this case, the degree of conservation is lower, but several residues are conserved or conservatively substituted. In particular, the overall “tunnel” shape of the active site observed in MPAO (18) is maintained in mSMO, although the central part of the tunnel is wider because of the substitutions T298W and T439W (numbering refers to MPAO). The substitution of Glu-62 involved in substrate binding in MPAO by a His residue in mSMO is the major difference observed between MPAO and mSMO active sites. This substitution is particularly interesting in that it can provide an explanation for the different pH profile of the activity observed in mSMO (see “Discussion”). Finally, it is interesting to note both in mSMO and in PAOh1 the conservation of Lys-300, a residue that has been hypothesized to play a structural role in the correct positioning of the FAD cofactor by forming an indirect hydrogen bond with FAD through a bridging water molecule (40).

**DISCUSSION**

Plant or animal recombinant PAOs have been never reported to be expressed in E. coli cells to our knowledge. This is the first report of a vertebrate polyamine oxidase overexpressed in secreted and secreted-tagged forms in such a heterologous system. SMO recombinant enzymes are targeted to the periplasmic membrane compartment, and the catalytically active proteins are expressed at a level of 6 units/liter of culture broth. The data obtained for the secreted recombinant mSMO perfectly match the ones obtained for the secreted-tagged enzyme form.

The analysis of the expressed mSMO protein gave an improved understanding of the biochemical features of this enzyme, since the purified recombinant enzyme was able to oxidize only Spm and failed to act upon Spd and the N'-acetyl derivatives. The enzyme specificity reported herein is in agreement with the one described in transfected human cells by Vujić et al. (30).

The precise nature of the reaction products and the cleavage position on the Spm substrate are those typical of an animal PAO enzyme. In line with this finding, the specific inhibition of mSMO enzyme activity was obtained with MDL72527 but not with pargyline.

The absorption spectrum of the native enzyme showed the typical three-banded spectrum for flavoproteins. Furthermore, the addition of the Spm substrate in anaerobic conditions resulted in a dramatic absorbance decrease, while reoxygenation of the enzyme restored the initial spectrum, indicating the involvement of a FAD group in the catalytic cycle.

An analysis of the molecular model of mSMO as compared with MPAO three-dimensional structure suggests that the catalytic tunnel of the former enzyme is wider. It is tempting to speculate that the preference for Spm over Spd as a substrate observed for mSMO is linked to the different shape of the catalytic tunnel in which the short Spd substrate would be bound in a “floppy” fashion, thus rendering less efficient the enzyme catalysis, which has been hypothesized to rely on an “in register” binding of the substrates.

The substitution of Glu-62 by a His residue in mSMO can provide an explanation for the peculiar pH dependence of the activity. In fact, it can be reasonably assumed that His-62 is partially protonated at pH values lower than 7.0. Thus, the binding of the cationic polyamine substrates would be unfavorable and enzyme activity would be very low as observed experimentally. At pH values higher than 7.0, His-62 would deprotonate, thus facilitating substrate binding and leading to an increase in enzyme activity, which is maximal at pH values of ~8.0.

In conclusion, in mammalian cells, polyamine catabolism seems to be mediated by the activity of two enzymes, PAO and the novel SMO, described in this work (30). The precise significance of both polyamine oxidase activities in the metabolism of polyamines remains to be established, particularly regarding the role of each enzyme in regulating polyamine concentrations in mammalian tissues in relation with growth processes and malignant transformation.

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