

Probing mammalian spermine oxidase enzyme–substrate complex through molecular modeling, site-directed mutagenesis and biochemical characterization

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Abstract Spermine oxidase (SMO) and acetylpolyamine oxidase (APAO) are FAD-dependent enzymes that are involved in the highly regulated pathways of polyamine biosynthesis and degradation. Polyamine content is strictly related to cell growth, and dysfunctions in polyamine metabolism have been linked with cancer. Specific inhibitors of SMO and APAO would allow analyzing the precise role of these enzymes in polyamine metabolism and related pathologies. However, none of the available polyamine oxidase inhibitors displays the desired characteristics of selective affinity and specificity. In addition, repeated efforts to obtain structural details at the atomic level on these two enzymes have all failed. In the present study, in an effort to better understand structure–function relationships, SMO enzyme–substrate complex has been probed through a combination of molecular modeling, site-directed mutagenesis and biochemical studies. Results obtained indicate that SMO binds spermine in a similar conformation as that observed in the yeast polyamine oxidase FMS1-spermine complex and demonstrate a major role for residues His82 and Lys367 in substrate binding and catalysis. In addition, the SMO enzyme–substrate complex highlights the presence of an active site pocket with highly polar characteristics, which may explain the different

substrate specificity of SMO with respect to APAO and provide the basis for the design of specific inhibitors for SMO and APAO.

Keywords Polyamines · Spermine oxidase · Molecular modeling · Site-directed mutagenesis · Enzyme–substrate complex

Introduction

The natural polyamines (PAs), putrescine (PUT), spermidine (SPD) and spermine (SPM), are ubiquitous, low molecular weight, aliphatic cations found in all eukaryotic cells (Pegg 2009; Cohen 1998; Wallace et al. 2003). The molecular functions of PAs involve reversible electrostatic interactions with nucleic acids, affecting chromatin status and gene expression, proteins, membranes and ion channels.

Polyamines affect cell growth, differentiation and apoptosis (Pegg 2009; Schipper et al. 2000; Thomas and Thomas 2001). Cells finely regulate the synthesis of PAs from precursors and PAs uptake from the diet, as well as inter-conversion, degradation and efflux (Persson 2009; Wallace et al. 2003). PA catabolism is dependent on the activity of spermidine/spermine N¹-acetyltransferase, which transfers acetyl groups from acetyl-coenzyme A to the N¹ position of either SPD (to produce N¹Ac-SPD) or SPM (to produce N¹Ac-SPM). N¹Ac-SPD and N¹Ac-SPM can be oxidized by the peroxisomal FAD-dependent enzyme N¹-acetylpolyamine oxidase (APAO) to produce, respectively, 1,3-diaminopropane and SPD, 3-acetoaminopropanal and H₂O₂. SPM can be also directly oxidized by spermine oxidase (SMO, first cloned by Wang et al. (2001) and originally named PAOh1), a flavoprotein of the polyamine oxidase family, which specifically recognizes

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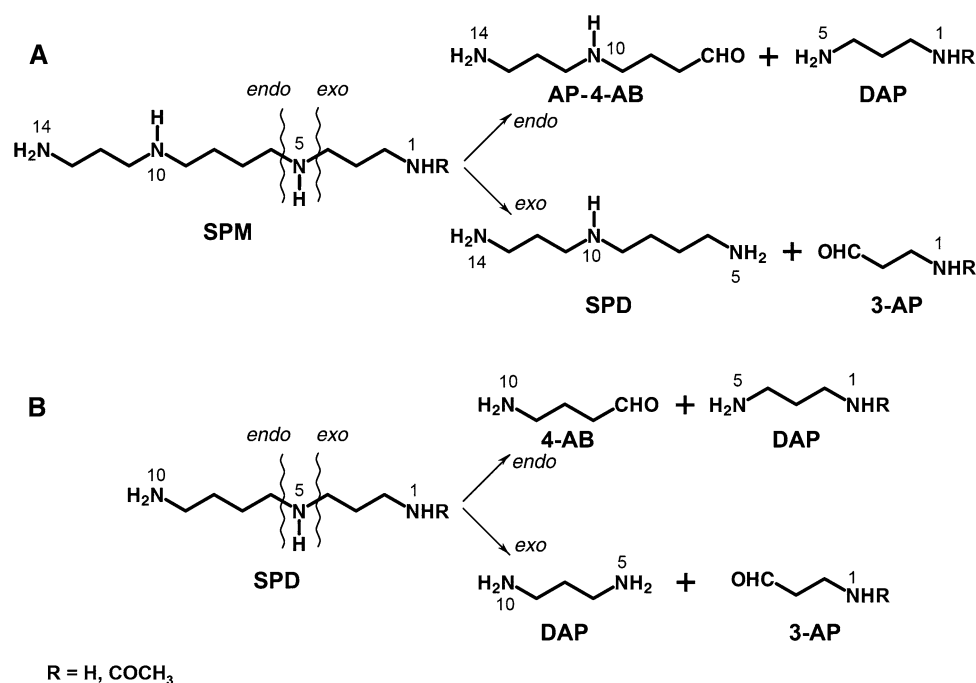


Fig. 1 Substrate oxidation mode in PAOs. APAO oxidizes the carbon on the exo side of the N⁵-nitrogens of N¹Ac-SPD and N¹Ac-SPM producing 1,3-diaminopropane (DAP) and SPD, respectively, in addition to 3-aceto-aminopropanal and H₂O₂ (**a** and **b** bottom reactions). SMO oxidizes the carbon on the exo side of the N⁵-nitrogen of SPM producing SPD, 3-aminopropanal (3AP) and H₂O₂ (**a** bottom reaction). ZmPAO oxidizes the carbon on the

side of the N⁵-nitrogens of SPD and SPM producing 4-aminobutylaldehyde (4AB) and 3-(aminopropyl)-4-aminobutylaldehyde (AP-4AB), respectively, in addition to 1,3-diaminopropane (DAP) and H₂O₂ (**a** and **b** top reaction). FMS1 oxidizes the carbon on the exo side of the N⁵-nitrogens of SPM, N¹Ac-SPM and N¹Ac-SPD (but not SPD) (**a** and **b** bottom reactions)

SPM as a substrate and produces SPD, 3-aminopropanal and H₂O₂ (Wang et al. 2001; Cervelli et al. 2003; Vujcic et al. 2002; Fig. 1).

Polyamines content is strictly related to cell growth, and dysfunctions in PAs metabolism have been linked with cancer (Casero and Pegg 2009; Gerner and Meyskens 2004; Thomas and Thomas 2003). Nowadays, the importance of the PA catabolic pathway in cancer therapeutics has been re-evaluated, as it has been shown to be involved in determining the cell response to PA analogs with anti-tumor activity (Amendola et al. 2009; Casero and Marton 2007; Casero et al. 2003; Pledgie et al. 2005). Thus, interest has been growing regarding the precise physiological role of the mammalian catabolic enzymes SMO and APAO. For instance, increasing evidences indicate that induction of SMO expression by inflammation or infectious agents has the potential to produce sufficient reactive oxygen species (ROS) in normal cells to potentially lead to carcinogenesis transformation (Babbar et al. 2007; Chaturvedi et al. 2004). In line with these assumptions, it has been observed that tissue from patients diagnosed with prostate cancer display locally increased SMO expression levels in the region of prostatic disease as compared to healthy individuals (Goodwin et al. 2008).

Several inhibitors of SMO and APAO have been studied, the most prominent example being MDL 72527 (N¹,N⁴-bis(2,3-butadienyl)-1,4-butanediamine), which inhibits FAD-containing polyamine oxidases (Bellelli et al. 2004; Bey et al. 1985; Bianchi et al. 2006; Wu et al. 2005). However, MDL 72527 does not show any selectivity between SMO and APAO (Bianchi et al. 2006), and inhibitors with strict selectivity for either SMO or APAO are not known (Bianchi et al. 2006).

In addition, from a structural viewpoint, no experimental data at the atomic level are available for SMO and APAO, which greatly hinders our ability to develop selective inhibitors needed to investigate in deeper detail the physiological role of these two enzymes. Atomic resolution three-dimensional structures are available only for two members of the polyamine oxidase family, namely the *Zea mays* polyamine oxidase (ZmPAO; Binda et al. 2001, 1999) and the yeast enzyme FMS1 (Huang et al. 2005). The two enzymes share the typical fold of FAD-dependent amine oxidases, characterized by an FAD binding domain and a substrate binding domain, whose contact interface defines the catalytic site (Binda et al. 1999). Interestingly, these two enzymes differ in substrate specificity and oxidation mode. In fact ZmPAO oxidizes the carbon on the

endo-side of the N⁵-nitrogens of SPD and SPM producing 4-aminobutyaldehyde and 3-(aminopropyl)-4-aminobutyaldehyde, respectively, in addition to 1,3-diaminopropane and H₂O₂ (Binda et al. 1999; Polticelli et al. 2005; Fig. 1). FMS1 oxidizes the carbon on the *exo* side of the N⁵-nitrogens of SPM, N¹Ac-SPM, N¹Ac-SPD and N⁸Ac-SPD (but not SPD; Landry and Sternglanz 2003). In the latter case, the products of SPM oxidation are SPD, 3-aminopropanal and H₂O₂ (Fig. 1). Thus, FMS1 mode of substrate oxidation is the same as observed for APAO and SMO (Wang et al. 2001; Cervelli et al. 2003; Vujcic et al. 2002; Wu et al. 2003).

Recently, in our laboratory, the mouse SMO (MmSMO) cDNA has been cloned and expressed in *Escherichia coli* BL21 DE3 cells, and the corresponding enzyme has been biochemically characterized (Bellelli et al. 2004; Cervelli et al. 2003). However, repeated attempts from independent laboratories to obtain protein crystals suitable for X-ray diffraction studies have been unsuccessful (A. Mattevi, personal communication; A. Fiorillo and A. Ilari, personal communication). On the other hand, multiple amino acid sequence alignment of mammalian SMOs with ZmPAO and FMS1 highlights a fairly high degree of sequence similarity (Fig. 2). In detail, MmSMO displays 41% sequence similarity with ZmPAO and 35% with FMS1. In addition, many of the residues building up the FMS1 active site and involved in enzyme–substrate interactions (Huang et al. 2005) are conserved in MmSMO (see “Results”). This observation, together with the common oxidation mode of the substrate (oxidation of the carbon on the *exo* side of the N⁵-nitrogens of the substrate), point to similar structural determinants for substrate binding in FMS1 and MmSMO.

Here, we have extended the study of MmSMO by probing the enzyme–substrate complex through a multidisciplinary approach involving molecular modeling, site-directed mutagenesis and biochemical studies. Results presented in this work show that MmSMO binds SPM in a similar conformation as that observed in the FMS1-SPM complex and highlight peculiar stereochemical characteristics of the MmSMO-SPM complex, which could be exploited for the design of inhibitor molecules selectively targeted to SMO or APAO.

Materials and methods

Molecular modeling of MmSMO and MmSMO-SPM complex

Two alternative methods were used to build MmSMO structural model, a homology modeling approach using the program NEST (Petrey et al. 2003) and the crystal structure

of ZmPAO (PDB code 1B5Q) (Binda et al. 1999) as a template, and a combined threading/*ab initio* modeling approach using the online server I-TASSER (Zhang 2008).

Regarding homology modeling, protein structures displaying significant sequence similarity with MmSMO were retrieved through two PSI-Blast (Schäffer et al. 2001) iterations against the PDB-derived sequence database using the sequence coded NP_663508 as a bait. The lowest E-value sequence retrieved by PSI-Blast was that of ZmPAO (E-value of 3×10^{-78}). Amino acid sequence alignment between the template ZmPAO and MmSMO was then obtained through multiple sequence alignment of the amino acid sequences of ZmPAO, FMS1 and mammalian SMOs using ClustalW (Larkin et al. 2007). This procedure yielded the alignment shown in Fig. 2. Based on this alignment, the molecular model of MmSMO was built using the program NEST (Petrey et al. 2003), a fast model building program that applies an “artificial evolution” algorithm to construct a model from a given template and alignment. The NEST option *tune 2* was used to refine the alignment avoiding the unlikely occurrence of insertions and deletions within template secondary structure elements. MmSMO molecular model was then stereochemically regularized through energy minimization in explicit solvent using CHARMM macromolecular mechanics package (Brooks et al. 1983), c33b1 version, and the CHARMM27 parameters and force field (MacKerell et al. 1998). The stereochemical quality of the model was evaluated using PROCHECK (Laskowski et al. 1993).

The MmSMO-SPM complex was built by manually docking SPM into MmSMO active site in the same conformation observed in the crystal structure of the FMS1-SPM complex. In detail, MmSMO molecular model was best fitted onto FMS1-SPM complex structure (rmsd 1.64 Å over 1084 superimposed atoms) and the SPM molecule was merged with the MmSMO model. The MmSMO complex has then been stereochemically regularized through energy minimization in explicit solvent using CHARMM macromolecular mechanics package (Brooks et al. 1983; MacKerell et al. 1998). The structural model of MmSMO-SPM complex is available in the PMDB database (<http://mi.caspar.it/PMDB/>) under the accession number PM0076222.

DNA methodology and construction of pMnSMO-HT expression plasmid

The methods described by Sambrook et al. (1989) were used for the extraction and manipulation of plasmid DNA and general DNA in *in vitro* methods. To clone the murine cDNA (GenBank accession number BC004831) by PCR amplification, the full-length cDNA was generated possessing modified 5' and 3' ends. In particular, the two

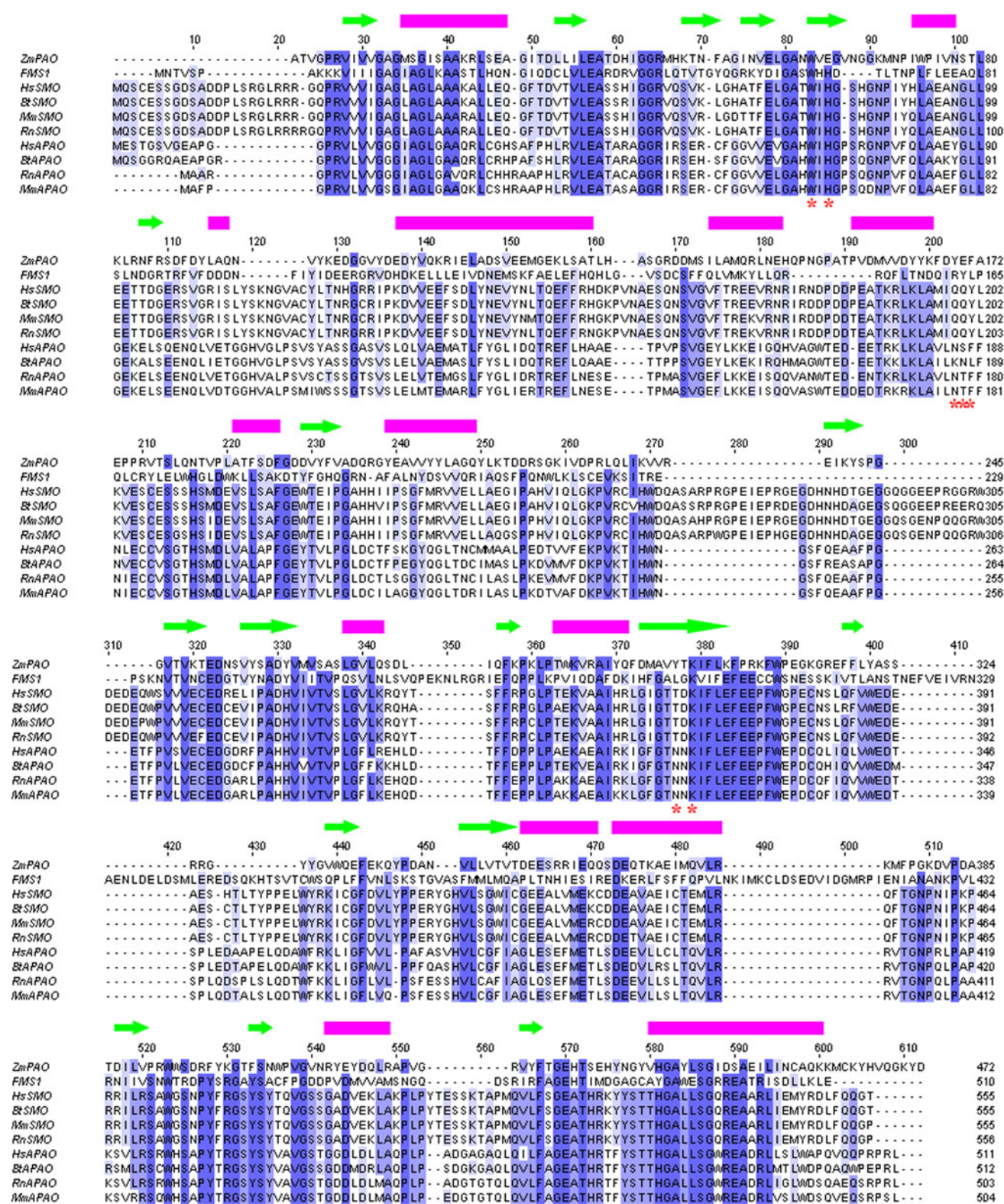


Fig. 2 Amino acid sequence alignment of ZmPAO, FMS1 and selected mammalian SMOs and APAOs. Green arrows and pink bars indicate secondary structure elements (β strands and α helices, respectively) of ZmPAO. Red stars indicate active site residues. Sequence blocks are colored according to BLOSUM62 score.

Sequence-specific numbering is given on the right end side of the figure. The alignment has been obtained using CLUSTALW2 (Larkin et al. 2007) and visualized using JalView (Waterhouse et al. 2009) (color figure online)

following synthetic oligonucleotides were used to introduce *NdeI* and *XhoI* restriction sites at the 5' and 3' ends of mSMO cDNA: SMO1-DIR, 5'-TTTCATATGCAAAGT TGTGAATCCAG-3' and SMO2-REV, 5'-AAATAT TCA ATGATGATGATGATGATGGGGCCCCTGCTGGAAG AGG-3', respectively. The amplified PCR product was restricted by *NdeI* and *XhoI* and ligated with the restricted *NdeI/XhoI* pET17b vector (Novagen), to obtain the genetic construct named pMmSMO-HT and containing the mature form of MmSMO protein joined to COOH-terminal HisTAG. The recombinant cDNA construct was sequenced to check the accuracy of the nucleotide sequence and then utilized to transform *E. coli* BL21 DE3 (Novagen) competent cells.

Site-directed mutagenesis of the SMO recombinant protein

To make mutants of the SMO protein, site-directed mutagenesis was carried out on the plasmid pMmSMO-HT using the QuikChange™ Site-directed Mutagenesis kit (Stratagene) and following the manufacturers' protocol. The mutagenic primer sequences used (Table 1), available upon request from the first author, produced the recombinant plasmid pMmSMO-HT-H82Q, pMmSMO-HT-H82E and pMmSMO-HT-K367M carrying the mutations described in the text. The introduction of the mutation was further confirmed by sequence analysis.

Expression and purification of MmSMO recombinant proteins in *E. coli* cells

The recombinant cDNA constructs pMmSMO-HT-H82Q, pMmSMO-HT-H82E, pMmSMO-HT-K367M and pMmSMO-HT-T528Y were used to transform *E. coli* BL21 DE3 cells. Induction and over-expression were carried out according to (Cervelli et al. 2004). The *E. coli* BL21 DE3 cells were harvested by centrifugation (4°C, 10 min, 10,000g), washed with 0.4 culture volume of 30 mM Tris–HCl (pH 8.0), containing 20% sucrose and 1 mM EDTA, and incubated for 5–10 min at room temperature. The

suspension was then centrifuged (10,000g for 10 min at 4°C) and the cell pellet resuspended in 5 mM imidazole, 0.5 M NaCl and 20 mM Tris–HCl (pH 7.9), and was sonicated. After centrifugation (12,000g for 30 min at 4°C), the soluble cell extract was purified using a slightly modified procedure of the His-Bind chromatography kit (Novagen). In particular, the supernatant was applied to a column (3-ml) with Ni²⁺ cations immobilized on the His-Bind resin (Novagen), and equilibrated with the binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl pH 7.9). The column was washed with binding buffer and then elution was performed with 200 mM imidazole, 0.5 M NaCl and 20 mM Tris–HCl of pH 7.9. The SDS/PAGE electrophoretic analysis was performed on purified recombinant mSMO proteins to assess the grade of purification.

CD spectroscopy

CD spectra were recorded at 25°C using a Jasco J-600 spectropolarimeter and quartz cells having 0.05 cm path length. Wild-type and mutant MmSMOs were solubilized in 5 mM sodium phosphate buffer, pH 8.0, at a concentration of about 0.2 mg/ml. Actual concentrations (~3 μM) were calculated by means of the 460 nm molar extinction coefficient ($\epsilon_{460} = 9,000 \text{ M}^{-1} \text{ cm}^{-1}$), which accounts for FAD absorption. Instrumental ellipticity θ (mdeg) was converted into mean residue molar ellipticity $[\theta]$ (deg cm²/dmol_{res}) by taking into account the cell path length and the protein concentration. For each sample, nine CD spectra in the far-UV region (190–250 nm) were recorded, averaged and smoothed. Secondary structures were estimated by using CONTIN, K2D and SELCON3 algorithms provided by the free software Dicroprot2000 (Deleage and Geourjon 1993).

Determination of MPAO catalytic parameters

The catalytic parameters (K_m and k_{cat}) for the oxidation of SPM by recombinant wild-type and mutant MmSMO enzymes were determined by following spectrophotometrically the formation of a pink adduct ($\epsilon_{515} = 2.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$),

Table 1 Primer sequences and plasmids used in this study

Primer	Sequence	Plasmid
SMOH82Q-F	5'-AGCCACCTGGATCCAGGGATCCCACGGGAAT-3'	pMmSMO-HT-H82Q
SMOH82Q-R	5'-ATTCCCGTGGGATCCCTGGATCCAGGTGGCT-3'	pMmSMO-HT-H82Q
SMOH82E-F	5'-AGCCACCTGGATCGAGGGATCCCACGGGAAT-3'	pMmSMO-HT-H82E
SMOH82E-R	5'-ATTCCCGTGGGATCCCTCGATCCAGGTGGCT-3'	pMmSMO-HT-H82E
SMOK367M-F	5'-TTGGTACCACTGACATGATCTTCCTTGAATT-3'	pMmSMO-HT-K367M
SMOK367M-R	5'-AATTCAAGGAAGATCATGTTCAGTGGTACCAA-3'	pMmSMO-HT-K367M
SMOT528Y-F	5'-GCAAGTACTACTCCTACACCCACGGTGCTCT-3'	pMmSMO-HT-T528Y
SMOT528Y-R	5'-AGAGCACCGTGGGTGTAGGAGTAGTACTTGC-3'	pMmSMO-HT-T528Y

as a result of the oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid catalyzed by horseradish peroxidase in 0.2 M sodium phosphate buffer at pH 8.5 and 25°C (Polticelli et al. 2005). k_{cat} values were calculated using saturating concentrations of SPM (4 mM) and keeping the O_2 concentration constant at the air-saturated level. K_{m} values for recombinant wild-type and mutant MmSMO for SPM were determined from Michaelis–Menten plots and nonlinear least-squares fitting of data was performed using the GraphPad Prism 4.0 software. Studies of the pH dependence of wild-type and mutant MmSMO activity were conducted in 0.2 M sodium phosphate buffer and in 100 mM sodium borate buffer at 25°C using saturating concentrations of SPM. Best fit of the experimental pH dependence curves was carried out with GraphPad Prism using the following equation to simulate a two- $\text{p}K_{\text{a}}$ s dissociation equilibrium:

$$A = A_{\text{max}}(-([H+]/(K_1 + [H+])) + ([H+]/(K_2 + [H+])))$$

where A is the catalytic activity of the enzyme at a given pH value, A_{max} is the maximum catalytic activity and K_1 and K_2 are the equilibrium dissociation constants. Catalytic parameters reported in Table 2 and Fig. 6 represent the average of at least three independent experiments each in duplicate.

Results

Modeling MmSMO enzyme–substrate complex

Several attempts were made to obtain crystals of the MmSMO enzyme without success (A. Mattevi, personal communication; A. Fiorillo and A. Ilari, personal communication), likely due to protein regions, which, by comparison with the ZmPAO amino acid sequence and secondary structure (Fig. 2), are predicted to be flexible surface loops. For this reason, details about the three-dimensional structure of MmSMO were obtained by

molecular modeling techniques. Two alternative methods were used to build MmSMO structural model, a homology modeling approach using the program NEST (Petrey et al. 2003) and the crystal structure of ZmPAO (PDB code 1B5Q; Binda et al. 1999) as a template, and a combined threading/ab initio modeling approach using the program I-TASSER (Zhang 2008). Figure 3 shows a comparison of the two models, which are highly consistent with each other, the rmsd between the two structures being only 0.93 Å over 1692 superimposed atoms. Differences between the two models are observed mainly in the conformation of surface loops, while all the residues building up the active site precisely match, with a C α rmsd of only 0.41 Å over 12 superimposed residues, giving essentially the same picture of the chemical environment of the MmSMO catalytic site and reinforcing the reliability of the predicted structural models. However, the homology modeled structure obtained using NEST appears more ordered as, consistent with the ZmPAO template, it displays a higher percentage of secondary structure (Fig. 3). In addition, overall G-value calculated for the latter model using PROCHECK (Laskowski et al. 1993) is -0.39 , well above the threshold of -0.5 for good quality models, and approximately 97% of residues were observed to lie in the allowed regions of the Ramachandran plot. Thus, further analyses were conducted on the homology-based structural model.

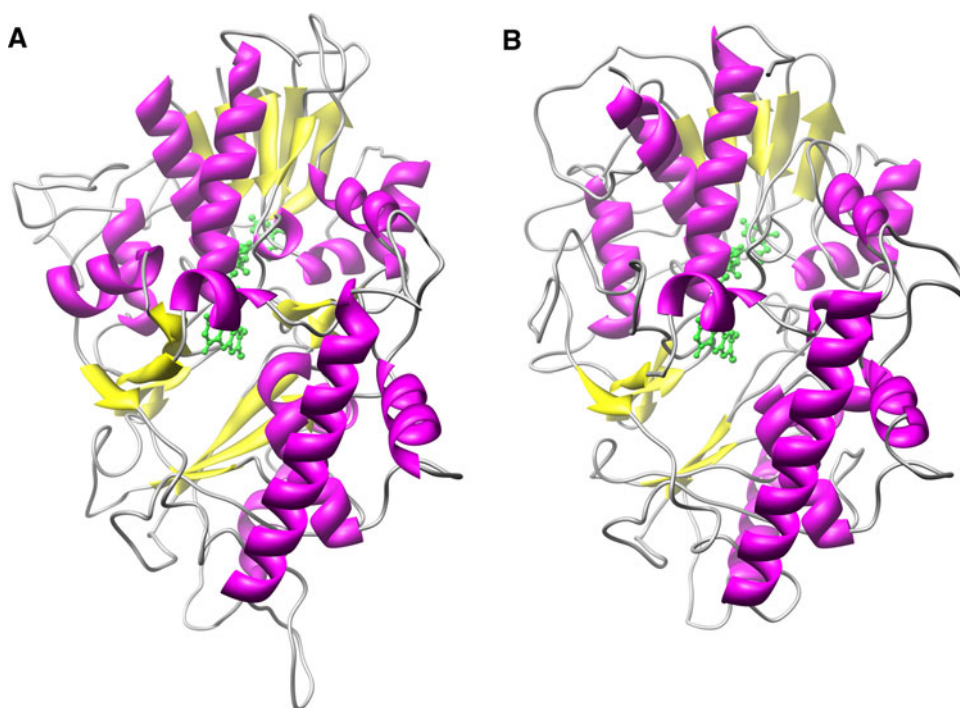
Analysis of MmSMO structural model highlights interesting characteristics of the active site shared with the yeast polyamine oxidase FMS1 (Fig. 4). In particular, MmSMO active site resembles that of FMS1 for the presence of His82 and Tyr482, which are conserved in FMS1 as His67 and Tyr450, while are substituted in ZmPAO by Glu62 and Phe403. It must be remembered that the only available enzyme–substrate complex for a PAO-like enzyme is the FMS1–SPM complex in which the substrate binds in a U-shaped conformation around the imidazole ring of His67. In addition, both MmSMO and FMS1 oxidize the exo carbon atom with respect to the secondary amino group of SPM as opposed to ZmPAO, which oxidizes the endo carbon atom (Fig. 1). Thus, SPM was docked into the active site of MmSMO structural model in the same conformation as that observed in the FMS1 enzyme–substrate complex, and the resulting complex was equilibrated in water through energy minimization. Analysis of the MmSMO–SPM complex, shown in Fig. 4, indicates that the substrate is bound with the C4 atom in the correct position to undergo oxidation (the distance between SPM C⁴ atom and FAD N⁵ atom being 4.9 Å in the model) through a series of polar interactions involving all the substrate amine groups. In detail, starting from the inner part of the catalytic site, the SPM N¹ atom is hydrogen bonded to Ser527 O and O γ atoms, the N⁵ atom is hydrogen bonded to Tyr482 OH atom and Gln200 O ϵ 1 atom, the N¹⁰ atom is

Table 2 Kinetic parameters determined for wild-type and mutant MmSMOs

MmSMO	k_{cat} (s^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)
Wild-type	3.57 ± 0.02	242 ± 18	0.015
H82Q	0.024 ± 0.01	530 ± 80	0.00005
H82E	Not detectable	Not detectable	/
K367M	0.10 ± 0.02	21 ± 5	0.005
T528Y	Not detectable	Not detectable	/

Catalytic parameters for the oxidation of SPM by recombinant wild-type and mutant MmSMOs were determined in 0.2 M sodium phosphate buffer at pH 8.5 and 25°C (for details see “Materials and methods”)

Fig. 3 Schematic representation of the molecular models of MmSMO obtained by (a) homology modeling using the program NEST (Petrey et al. 2003) and the three-dimensional structure of ZmPAO (PDB code 1B5Q) (Binda et al. 1999) as a template, and (b) by the ab initio automatic modeling program I-TASSER (Zhang 2008)



hydrogen bonded to His82 N ϵ 2, while the N¹⁴ atom is hydrogen bonded to Glu224 O atom. MmSMO structural model predicts an important role for His82 in the binding of the substrate. Furthermore, MmSMO His82 is conserved both in FMS1 and APAOs, proteins which share the exo carbon oxidation mode. Another interesting feature of MmSMO model is the presence of Thr528 in the place of the bulkier Tyr439 found in ZmPAO (Fig. 4), a residue which has been implicated in SPM correct binding for endo carbon oxidation (Binda et al. 2001). In particular, the presence of a Tyr residue in position 528 in MmSMO would not be compatible with SPM binding in the position predicted by our model, as the phenol ring of the Tyr residue would occupy the position of the N¹ and C² atoms of SPM, thus preventing substrate binding (data not shown). Finally, MmSMO, as all the polyamine oxidase like enzymes, displays the conservation of Lys367, orthologous to Lys300 residue forming in ZmPAO the Lys–H₂O–FAD structural motif, which has been shown to be essential for catalysis in the latter protein (Polticelli et al. 2005).

Characterization of wild-type and mutant MmSMOs

To experimentally validate the MmSMO-SPM complex structural model discussed above, and to characterize the MmSMO catalytic properties, residues His82, Lys367 and Thr528 were selected for site-directed mutagenesis experiments. In detail, His82 was substituted with a Gln residue, (H82Q), which partly preserved the hydrogen bonding

properties of His, and with a Glu residue (H82E) present in orthologous position in ZmPAO. Lys367 was substituted with the isosteric neutral Met residue (K367M) and Thr528 with the bulkier Tyr residue (T528Y) present in orthologous position in ZmPAO.

Recombinant wild-type and mutant MmSMOs were produced by heterologous expression in *E. coli* BL21 DE3 cells. Proteins were purified by His-Bind chromatography and their purity was assessed by SDS/PAGE electrophoretic analysis (not shown). Kinetic analysis of wild-type and mutant MmSMOs was then carried out to characterize their catalytic features (Table 2). Mutations H82Q and K367M lead to a 150 and 36-fold reduction of k_{cat} , respectively, while MmSMO mutants H82E and T528Y resulted to be inactive enzymes. The K_{m} value increases about twofold in H82Q, in line with a role of this residue in the binding of the substrate, while the opposite is observed in K367M, probably as a consequence of the reduced repulsion between the basic substrate and the enzyme active site. The absence of any detectable activity in the T528Y mutant is in agreement with the structural model of the MmSMO-SPM complex proposed in this work, in that it suggests that introduction of a bulky side chain at position 528 prevents substrate binding due to steric repulsion. Mutation H82E, mimicking ZmPAO active site, probably leads to an out-of-register binding of the substrate (Binda et al. 2001), resulting in an inactive enzyme. This is in line with the different substrate oxidation mode of ZmPAO (which oxidizes the endo carbon atom adjacent to the secondary amino group of SPM; Binda et al. 1999) with

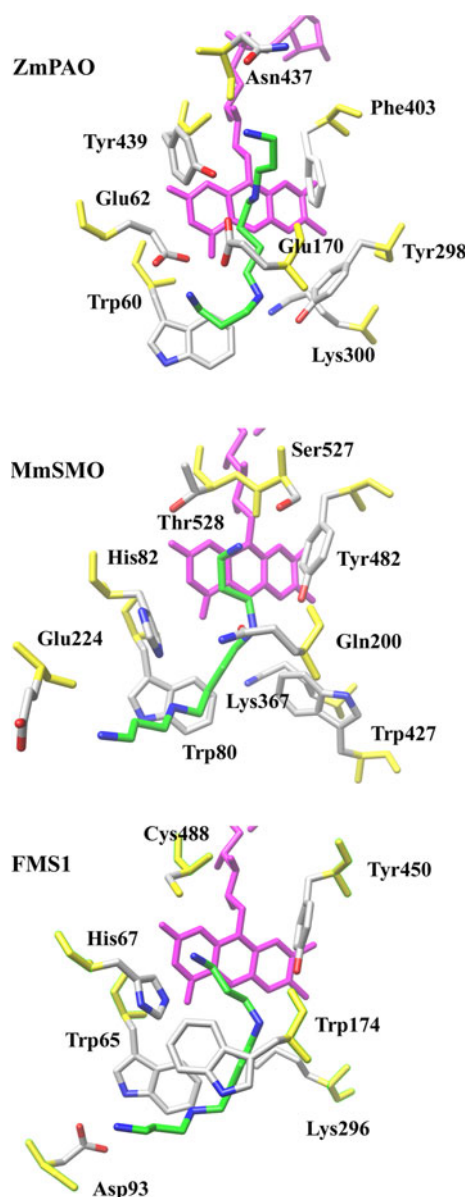


Fig. 4 Comparison of the enzyme–substrate interactions in ZmPAO (Polticelli et al. 2005), MmSMO (present work) and FMS1 (PDB code 1XPQ; Huang et al. 2005). For clarity, spermine carbon atoms are colored in green, the FAD moiety in magenta and the backbone atoms in yellow. The structural model of MmSMO-SPM complex is available in the PMDB database (<http://mi.caspur.it/PMDB/>) under the accession number PM0076222 (color figure online)

respect to MmSMO (which oxidizes the exo carbon atom adjacent to the secondary amino group of SPM; see Fig. 1; Cervelli et al. 2003; Wang et al. 2003).

To rule out that major structural rearrangements could contribute to the results obtained, circular dichroism spectra were recorded for recombinant wild-type and mutant enzymes. Results obtained indicate that wild-type and mutant proteins share essentially the same structural properties (Fig. 5). In addition, optical spectra in the FAD

absorbing region are practically superimposable for wild-type and mutant MmSMOs (data not shown), further confirming the correct folding and cofactor binding in all the proteins under study.

The pH dependence of the catalytic activity has also been studied for the wild-type and the two active mutant enzymes. Wild-type MmSMO enzymatic activity increases in the pH range 6.5–8.5, reaching a maximum at pH 8.5 and decreasing for higher pH values (Fig. 6). The pH dependence of the catalytic activity of H82Q is essentially unchanged with respect to the wild type, while a slight shift toward higher pH values is observed for the K367M mutant (Fig. 6; Table 3).

pK_as calculations

To further investigate the identity of the ionizable group(s) controlling the pH dependence of the catalytic activity of MmSMO, pK_a values were calculated using the PROPKA software (Bas et al. 2008), for the ionizable active site residues and substrate groups in the substrate-free and substrate-bound wild-type MmSMO (Table 4). Results obtained predict that His82 deprotonates at very acidic pH values, while Lys367 deprotonation is predicted to occur at approximately pH 6.0. Thus, both residues would be already deprotonated in the pH range 6.5–8.5 in which the pH-dependent increase of MmSMO catalytic activity occurs. Interestingly, the N¹ and N⁵ substrate amino groups located in the vicinity of FAD, the deprotonation of which is considered essential for catalytic activity to occur (Henderson Pozzi et al. 2009; Polticelli et al. 2005), are predicted to deprotonate at approximately pH 7.5 and 7.0, respectively, consistent with deprotonation of the substrate being responsible for the observed pH dependence of the catalytic activity.

Discussion

Polyamines affect cell growth, differentiation and apoptosis, and PA metabolism defects have been linked to cancer by a number of independent studies (Amendola et al. 2009; Casero and Marton 2007). It is thus highly desirable to have specific inhibitors of SMO and APAO to be able to analyze their precise role in PA metabolism. However, none of the available PAO inhibitors displays the desired characteristics of selective affinity and specificity (Bianchi et al. 2006).

In this work, given the difficulties encountered in the experimental structure determination of MmSMO, we have attempted to obtain structural information regarding the enzyme–substrate complex through molecular modeling techniques and validation of the model obtained by means

Fig. 5 Circular dichroism spectra of wild-type and mutant MmSMOs. For experimental details see “Materials and methods”

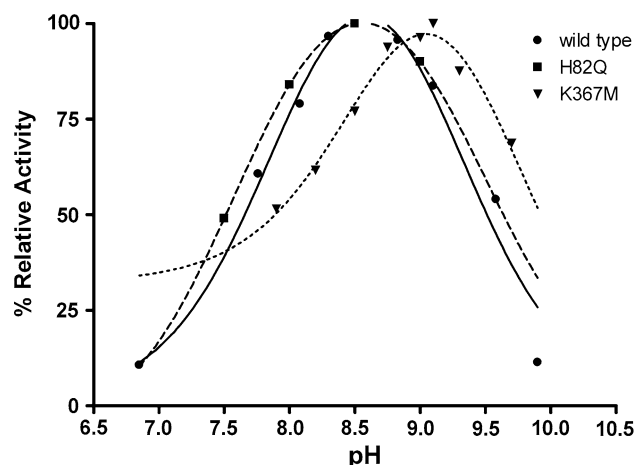
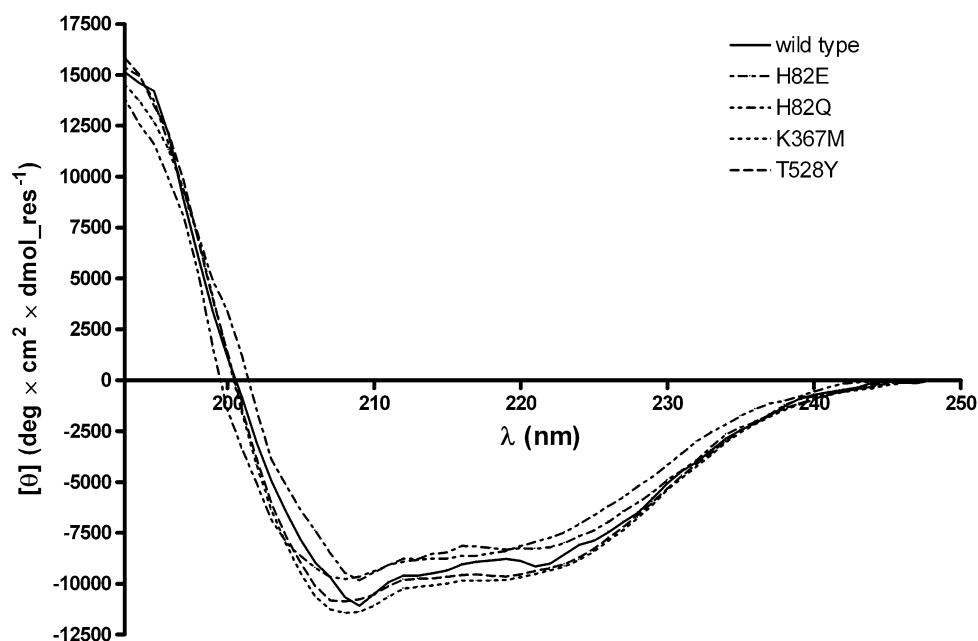


Fig. 6 pH dependence of the catalytic activity of wild-type (circles), H82Q (squares) and K367M (triangles) MmSMOs. For experimental details see “Materials and methods”

Table 3 pK_a values of the pH dependence of the catalytic activity of wild-type and mutant MmSMOs

	Wild type	H82Q	K367M
pK_1	7.98 ± 0.22	7.61 ± 0.22	8.67 ± 0.24
pK_2	9.19 ± 0.17	9.46 ± 0.17	9.59 ± 0.16
R^2	0.97	0.97	0.98

of site-directed mutagenesis and functional characterization of MmSMO variants. The model presented in this paper is consistent with a binding mode of SPM within MmSMO active site similar to that observed in the FMS1-SPM complex (Fig. 4; Huang et al. 2005). In particular, a relevant role in the binding of SPM is predicted for His82,

Table 4 Calculated pK_a values for ionizable active site residues and substrate groups for MmSMOs

Residue	wt	wt-SPM
Asp104	1.97	1.97
Glu106	4.01	4.01
Glu224	4.50	4.50
Glu391	6.62	6.62
His82	2.81	<0.0
His530	1.02	0.12
Tyr201	>14.0	>14.0
Tyr482	>14.0	>14.0
Tyr484	>14.0	>14.0
Tyr526	10.28	10.41
Lys367	5.90	5.76
Spm (N14)		9.18
Spm (N10)		7.47
Spm (N5)		6.99
Spm (N1)		7.48

Selected ionizable active site residues are those within 8 Å distance from the substrate. pK_a values were calculated using PROPKA (Bas et al. 2008)

which is hydrogen bonded to one of the SPM N¹⁰ hydrogen atoms. Indeed, mutation of His82 to Gln or Glu greatly reduces or even abolishes (in the latter case) MmSMO activity, confirming that this residue is essential for correct SPM binding within the MmSMO active site. The effect of His82Gln mutations seems to be limited to SPM binding mode, leading to a 150-fold decrease of k_{cat} , and not to affect either the enzyme affinity for the substrate (K_m value is only doubled in the mutant with respect to the wild type;

Table 2) or the catalytic mechanism, since the pH dependence of the catalytic activity is essentially unchanged with respect to the wild-type enzyme (see Table 3; Fig. 6). Another feature of the MmSMO-SPM complex is that SPM binding would not be compatible with the presence of a bulky residue in position 528, at variance with what is observed for ZmPAO, in which a Tyr residue (Tyr439) is present in orthologous position (see Fig. 4). In agreement with the proposed model, mutation of Thr528 to Tyr yields an inactive enzyme, a fact that seems to be justified only by the steric hindrance of the bulky Tyr side chain, which would partly occlude the SPM binding pocket.

An interesting structural feature shared by MmSMO with many other FAD-dependent amine oxidases such as ZmPAO, FMS1, L-amino acid oxidase, monoamine oxidase B, monomeric sarcosine oxidase and lysine specific demethylase 1 (LSD1) is the presence of a Lys residue in the vicinity of the FAD cofactor, which, in the structurally characterized enzymes, has been shown to form a Lys-H₂O-FAD structural motif (Binda et al. 1999, 2002; Huang et al. 2005; Stavropoulos et al. 2006). This structural motif has been shown to be essential for the catalytic activity in ZmPAO and LSD1 (Polticelli et al. 2005; Stavropoulos et al. 2006), while only a limited decrease of the activity has been observed upon mutation of the Lys residue in APAO (Henderson Pozzi et al. 2009). In this regard, mutation of Lys367 in MmSMO leads to a substantial decrease of the activity with respect to the wild-type enzyme, indicating that this residue, as already observed in ZmPAO, plays a relevant role in the catalytic mechanism of MmSMO. Characterization of ZmPAO Lys300Met mutant, which displays a 1400-fold reduction in the rate of flavin reduction, led to the hypothesis that Lys300 could have the properties of a strong base at neutral pH (Polticelli et al. 2005). In addition, analysis of the solvent isotope effect (SIE) in stopped-flow and steady-state turnover studies of wild-type ZmPAO gave support for a role of the water molecule forming the Lys-H₂O-FAD structural motif in catalysis. In fact, stopped-flow analysis revealed that a sizeable SIE (SIE = 5) accompanied flavin reduction, and an SIE of value 2.3 was also observed in steady-state turnover of the wild-type enzyme (Polticelli et al. 2005). According to the results of pK_as calculation shown in Table 4, this could be the case also in MmSMO. In fact, Lys367 pK_a value is predicted to be below pH 6.0 and thus this residue could play a similar role, though less critical, to that of Lys300 in ZmPAO. It must be recalled that also in the case of LSD1, mutation of the Lys residue forming the Lys-H₂O-FAD structural motif (Lys661) leads to a dramatic decrease (>95%) of catalytic activity (Stavropoulos et al. 2006).

It may be argued that the effects of Lys367 mutation in MmSMO and Lys300 mutation in ZmPAO could be due to

some kind of structural rearrangement of the protein and/or of the environment and conformation of the FAD cofactor. However, structural characterization of the ZmPAO Lys300Met mutant rules out this hypothesis at least as far as ZmPAO is concerned. In fact, the three-dimensional structure of Lys300Met ZmPAO is practically identical to that of the wild-type enzyme, the backbone rmsd between the two structures being only 0.39 Å and the FAD position and conformation perfectly superimposable (A. Fiorillo, manuscript in preparation).

From the data presented in this work, it must be concluded that there are differences in the mechanistic details of polyamine oxidation between MmSMO and APAO, despite their similarity in substrate oxidation mode (oxidation of the exo carbon atom), as only a minor decrease of the catalytic activity has been observed upon mutation of the Lys367 orthologous residue in APAO (Henderson Pozzi et al. 2009).

Analysis of the structural details of MmSMO active site model highlights interesting features, which can shed light on the different substrate specificity of MmSMO and APAO. In fact, docking of N¹Ac-SPM into MmSMO active site indicates that the methyl group of N¹Ac-SPM would be located in a highly polar pocket made up of the side chains of Glu216 and Ser218 (see Supplementary materials, Figure S1). This is clearly energetically unfavorable leading to the hypothesis that SMOs exploit this feature to selectively bind SPM and not N¹Ac-SPM. Interestingly, Glu216 and Ser218 are substituted in APAO by a Leu and Ala residue, respectively (Fig. 2), making the corresponding pocket in APAO perfectly fit to host the methyl group of the acetylated polyamine. Site-directed mutagenesis experiments are being carried out to confirm this hypothesis, which would form the structural basis for the design of selective inhibitors of SMOs and APAOs.

The differences in substrate specificity between SMO and FMS1 also deserve some comments. In fact, both proteins oxidize SPM and not SPD, but FMS1 is also active on the acetylated polyamines N¹Ac-SPM, N¹Ac-SPD and N⁸Ac-SPD. Comparative analysis of the SMO molecular model described in this work and of the crystal structure of FMS1 reveal that the active site of the latter protein is much wider than that of SMO and that the highly polar pocket made up of Ser216 and Glu218 in SMO is not present in FMS1. This probably explains the ability of FMS1 to bind and oxidize acetylated polyamines at variance with SMO. The lack of activity on SPD for both proteins is more puzzling, but it could be due to electrostatic factors. In fact, deprotonation of the N¹ and N⁵ substrate amino groups located in the vicinity of FAD is considered essential for catalytic activity to occur (Henderson Pozzi et al. 2009; Polticelli et al. 2005). In the case of SPM, the interaction of the N¹⁴ amino

group with an acidic residue (Asp93 in FMS1 and Glu224 in SMO; Fig. 4) keeps this group protonated well above neutral pH, as confirmed by pK_a s calculations shown in Table 4, and facilitates the deprotonation of the N¹ and N⁵ amino groups. No such interaction would be possible with the shorter SPD molecule, which in turn would lead to a higher basic character of the N¹ and N⁵ amino groups, making SPD a very poor substrate for the two proteins.

Conclusions

It is well known that in mammals, PAs affect cell growth, differentiation and apoptosis, and PA metabolism defects have been linked to cancer (Amendola et al. 2009; Casero and Marton 2007). It is thus highly desirable to have specific inhibitors of SMO and APAO enzymes to analyze their precise role in PAs metabolism. The results presented in this paper, exploiting the synergy between molecular modeling, site-directed mutagenesis and biochemical characterization, provide a molecular view of the enzyme–substrate interactions in MmSMO and represent a first step toward the design of new SPM-derived inhibitors. In detail, SPM is predicted to be bound in MmSMO active site through interactions with Ser527, Tyr482, Gln200, His82 and Glu224. In addition, we have shown that His82 and Lys367 play an important role in MmSMO catalytic activity. His82 is most likely involved in the correct binding of SPM, as already observed for the orthologous His67 residue in FMS1, while a direct participation of this residue in the catalytic mechanism seems unlikely as its mutation to Gln does not lead to a significant change of the pH profile of the activity. On the contrary, the results obtained point to a direct involvement of Lys367 in MmSMO catalytic mechanism as its mutation to Met causes a substantial decrease of k_{cat} and a shift of the pH profile of the activity. In this regard, pK_a s calculations support the hypothesis that substrate deprotonation, facilitated by electrostatic interactions with Lys367, is responsible for the observed pH dependence of the activity. Finally, the MmSMO-SPM model highlights the presence of an active site pocket, which displays highly polar characteristics in MmSMO (being formed by a Glu and a Ser residue) and hydrophobic characteristics in APAO (being formed by a Leu and a Val residue). This difference may explain the different substrate specificity of the two enzymes and provide the basis for the design of specific inhibitors for SMO and APAO.

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