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Original Contribution

A novel enzyme with spermine oxidase properties in bovine liver mitochondria: Identification and kinetic characterization

Emanuela Bonaiuto [a], Silvia Grancara [b], Pamela Martinis [b], Annarita Stringaro [c], Marisa Colone [c], Enzo Agostinelli [d], Alberto Macone [d], Roberto Stevanato [e], Fabio Vianello [f,g], Antonio Toninello [b,*], Maria Luisa Di Paolo [a,h,**]

[a] Department of Molecular Medicine, University of Padova, Via G. Colombo 3, 35131 Padova, Italy
[b] Department of Biomedical Sciences, University of Padova, Via G. Colombo 3, 35131 Padova, Italy
[c] Department of Technology and Health, Italian Institute of Health, 00161 Roma, Italy
[d] Istituto Pasteur-Fondazione Cenci Bolognetti, Department of Biochemical Sciences "A. Rossi Fanelli," Sapienza University of Rome and Institute of Biology and Molecular Pathology, Italian Research Council, P.le Aldo Moro 5, 00185 Roma, Italy
[e] Department of Molecular Science and Nanosystems, Università Ca' Foscari, Dorsoduro 2137, 30123 Venezia, Italy
[f] Department of Comparative Biomedicine and Food Science, Polo Agripolis, Viale dell'Università 16, University of Padova, 35020 Legnaro, Italy
[g] Regional Centre for Advanced Technologies and Materials, Department of Physical Chemistry, Palacky University in Olomouc, 17 Listopadu 1192/12, 771 46 Olomouc, Czech Republic
[h] Consorzio Interuniversitario "Istituto Nazionale Biostrutture e Biosistemi," Viale delle medaglie d'Oro 305, 00136 Roma, Italy

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ABSTRACT

The uptake of spermine into mammalian mitochondria indicated the need to identify its catabolic pathway in these organelles. Bovine liver mitochondria were therefore purified and their capacity for natural polyamine uptake was verified. A kinetic approach was then used to determine the presence of an MDL 72527-sensitive enzyme with spermine oxidase activity in the matrix of bovine liver mitochondria. Western blot analysis of mitochondrial fractions and immunogold electron microscopy observations of purified mitochondria unequivocally confirmed the presence of a protein recognized by anti-spermine oxidase antibodies in the mitochondrial matrix. Preliminary kinetic characterization showed that spermine is the preferred substrate of this enzyme; lower activity was detected with spermidine and acetylated polyamines. Catalytic efficiency comparable to that of spermine was also found for 1-aminododecane. The considerable effect of ionic strength on the V_{max}/K_M ratio suggested the presence of more than one negatively charged zone inside the active site cavity of this mitochondrial enzyme, which is probably involved in the docking of positively charged substrates. These findings indicate that the bovine liver mitochondrial matrix contains an enzyme belonging to the spermine oxidase class. Because H_2O_2 is generated by spermine oxidase activity, the possible involvement of the latter as an important signaling transducer under both physiological and pathological conditions should be considered.

Abbreviations: ACRL, acrolein; AMA, aminoaldehyde; AO, amine oxidase; BLM, bovine liver mitochondria; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, 2-({2-[bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino)acetic acid; FAD, flavin-adenine-dinucleotide; EPPS, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid; MPT, mitochondrial permeability transition; MMAO, mitochondrial matrix amine oxidase; MAO, monoamine oxidase; APAO, N^1-acetylpolyamine oxidase; AcSpd, N^1-acetylspermidine; AcSpm, N^1-acetylspermine; MDL 72527, N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine; "not purified, " from mitochondria isolated but not purified through Ficoll gradient, n.d., not detectable, PMP70, 70-kDa peroxisomal membrane protein; PUT, putrescine; RLM,, rat liver mitochondria; ROS, reactive oxygen species; RCI, respiratory control index; SSAO, semicarbazide-sensitive amine oxidase; SPD, spermidine; SSAT, spermidine/spermine N^1-acetyltransferase; SPM, spermine; SMO, spermine oxidase; TETA, triethylenetetramine.

* Corresponding author.
** Corresponding author at: Department of Molecular Medicine, University of Padova, Via G. Colombo 3, 35131 Padova, Italy. Fax: +39 049 8073310.
E-mail addresses: antonio.toninello@unipd.it (A. Toninello), marialuisa.dipaolo@unipd.it (M.L. Di Paolo).

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Introduction

Naturally occurring polyamines, found in virtually all living cells, are positively charged molecules at physiological pH. They play critical roles in many cell functions, including nucleic acid and protein synthesis, gene expression, protein function, scavenging of reactive oxygen species, regulation of ion channels, and protection of macromolecular structures. Maintenance of the proper concentration of polyamines is necessary for these functions, whereas excess in their levels can lead to toxicity [1–3]. Toxic effects are also the result of polyamine catabolism due to generation of reactive oxygen species (ROS) by a variety of oxidases. Some of these, such as diamine oxidase and serum amine oxidase, are well known, whereas others, such as polyamine oxidases and spermine oxidase (SMO), have only recently been isolated. The mechanistic aspects of these oxidase activities, excluding SMO, are quite well known, and their cofactors and preferred polyamines as substrates have been characterized. Amine oxidases can be classified as Cu^{2+} containing (or semicarbazide sensitive), such as diamine oxidases, vascular-adhesion protein-1 and lysiloxidases, and FAD-dependent AOs (including monoamine, polyamine, and spermine oxidases; see Table 1 in [4]).

As often reported, spermine and other polyamines are transported in mammalian mitochondria by an energy-dependent mechanism, having electrical membrane potential $\Delta\Psi$ as its driving force [1]. As a result of this transport, spermine exhibits several significant effects for mitochondrial bioenergetic functions [5], particularly at the level of the mitochondrial permeability transition (MPT), a phenomenon related to intrinsic apoptosis. Spermine uptake into the mitochondrial matrix also indicates the need to identify a catabolic pathway in these organelles. However, despite great effort in this direction, for many years no definitive results have been forthcoming. It should be noted that polyamine oxidase activity has been found in rat tissues, particularly in liver, in which the highest activity was detected in the light mitochondrial fraction [6], most of the lysosomes and peroxisomes, and intact Golgi membranes. However, the first preliminary kinetic characterization of a copper-containing (semicarbazide-sensitive) amine oxidase in the liver mitochondrial matrix was only reported in 2009, with putrescine and spermine as substrates [7].

Among the polyamine oxidases belonging to the family of flavoprotein amine oxidases, spermine oxidase (SMO, originally named PAO1) is highly specific for spermine, which is directly oxidized by SMO to produce spermidine, H_2O_2, and 3-aminopropanal with no previous acetylation step [8–10]. SMO shows very low catalytic activity vs N^1-acetylspermine, a substrate of acetylpolyamine oxidases (APAO) [11]. At present, there are no very potent and selective SMO inhibitors [10]: among tested compounds, the APAO inhibitor N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527) can also inhibit SMO and, with the polyamine analogs BENSpm, CPENSpm, are the most commonly used compounds in inhibition studies in cell systems. At present, no three-dimensional crystal structures of mammalian SMO or APAO have been resolved, and molecular models and site-directed mutagenesis studies have been used to obtain information on the role of some residues in determining the difference in substrate specificity [12,13].

The SMO gene codes for multiple splice variants, and two catalytically active isoforms have been found in both cytosol and nucleus (SMO1 and SMO 5 in human and SMOμ in mouse). In mouse, isoform SMOα is located only in the cytoplasm [11,14]. In addition, although polyamine oxidases are constitutively expressed, SMO, which is normally present in mammalian cells at low levels, is highly inducible by a variety of stimuli, such as *Helicobacter pylori* infection (in gastric cancer), the proinflammatory TNF-alpha (in lung epithelial cells) [15], and polyamine analogs [8,16]: that is, SMO is associated with several pathological conditions.

The observation that polyamines are transported in mitochondria by an energy-dependent mechanism [1] thus reinforces the hypothesis that mitochondria contain an enzyme for the catabolic pathway of spermine. In this study we report the identification of an enzyme with spermine oxidase activity in bovine liver mitochondria.

Table 1
Markers of mitochondrial and cytosolic fractions from bovine liver.

Fractions from bovine liver	Lactate dehydrogenase activity ($nmol_{NADH}\ min^{-1}\ mg^{-1}_{prot}$)	Citrate synthase activity ($nmol_{CoASH}\ min^{-1}\ mg^{-1}_{prot}$)
Homogenate	84.2 ± 15.5	0.96 ± 0.3
Cytosol fraction	266.2 ± 12.5	3.4 ± 0.8
Mitochondria (before Ficoll gradient)	35.0 ± 3.5	10.8 ± 1.8
Mitochondria	n.d.[a]	29.0 ± 1.2
Mitochondrial soluble matrix	n.d.[a]	183.8 ± 13.7
Mitochondrial membranes	2.0 ± 0.5	12.8 ± 1.2

[a] Not detectable (activity < 0.2 $nmol_{NADH}\ min^{-1}\ mg^{-1}_{prot}$).

Materials and methods

Materials

All chemicals, of the highest commercially available purity, were purchased from Fluka and Sigma-Aldrich S.r.l. (Milan, Italy), with the exception of Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), from Molecular Probes/Invitrogen (Invitrogen s.r.l, San Giuliano Milanese (MI, Italy). MDL 72527 came from Marion Merrell Dow Research Institute (Strasbourg, France). For GS-MS analysis, diethyl ether, ethyl acetate, and dichloromethane were of pesticide grade; sodium chloride (Fluka) was washed successively with methanol, acetone, dichloromethane, and diethyl ether, followed by drying under vacuum (100 °C, 1 h) before being used.

Fresh bovine liver was collected from a local abattoir. Polyclonal anti-PMP70 antibodies produced in rabbits were purchased from Sigma-Fluka, and polyclonal spermine oxidase antibodies were a kind gift from Professor Paolo Mariottini (Università di "Roma Tre" Rome, Italy). Antisera were obtained by the intradermal injection into New Zealand rabbits of 2 ml emulsion 1:1 mSMOα (2 mg/ml): complete Freund's adjuvant suspensions, boosting being carried out at 2-week intervals. A γ-globulin fraction was obtained from the antisera by a single precipitation with 33% $(NH_4)_2SO_4$. The anti-SMO antibodies were purified from the γ-globulin fraction through an affinity column prepared by coupling the SMO recombinant protein [17] to cyanogen bromide-activated Sepharose 4B, according to the manufacturer's instructions (Sigma-Aldrich). The anti-mSMO antibodies were eluted with 0.1 M HCl/glycine, pH 3.0, and 0.5 M NaCl, and then neutralized with 0.5 M Na_2HPO_4 to obtain a final pH of 6.0. The immunoreactivity of the final γ-globulin preparation against the SMO protein was tested by Western blot analysis (data not shown).

A Cary-Eclipse fluorimeter (Varian Inc., Palo Alto, CA, USA) and a Cary 50 Scan UV-visible spectrophotometer (Varian Inc.) were emplo yed for fluorescence and spectrophotometric measurements, respectively. Kodak Image Station 4000 mm PRO was employed for acquiring the chemiluminescent images of Western blots.

Mitochondrial isolation and purification

Bovine liver was homogenized in isolation medium (250 mM sucrose, 5 mM Hepes, 0.5 mM EGTA, pH 7.4) and subjected to centrifugation (900 *g*) for 5 min. The supernatant was centrifuged at 12,000 *g* for 10 min to precipitate crude mitochondrial pellets. The pellets were resuspended in isolation medium plus 1 mM ATP and