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ORIGINAL ARTICLE

Do mammalian amine oxidases and the mitochondrial polyamine transporter have similar protein structures?

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Abstract Polyamine transport across the mitochondria membrane occurs by a specific, common uniporter system and appears controlled by electrostatic interactions as for polyamine oxidative deamination by bovine serum and mitochondrial matrix amine oxidases was found. In fact in all the cases, while the catalytic constants or the maximum uptake rate values show little changes with the number of the positive charges of the substrates, Michaelis–Menten constant values demonstrate exponential dependence, confirming that electrostatic forces control the docking of the substrate into the enzyme active site or polyamine channel. By the treatment of the kinetic data in terms of Gibbs equation or Eyring theory, the contribution of each positive charge of the polyamine to the Gibbs energy values for the oxidative deamination of polyamines by two mammalian amine oxidase and for polyamine transport, are obtained. These values were comparable and in good accordance with those reported in literature. Previous studies demonstrated that two negative functional groups in the active site of bovine serum and mitochondrial matrix amine oxidases interact electrostatically with three positive charges of the polyamines in the formation of the enzyme–substrate complex. Remembering the structure–function relationship of proteins, our results suggest analogous interactions in the polyamine transporter and, as a consequence, a partial structural similitude between two proteins. It follows that the primary sequences of the amino oxidases and the mitochondrial transport may, in part, be conserved.

Keywords Amine oxidases · Mitochondrial polyamines uniporter · Polyamines

Abbreviations

BSAO	Bovine serum amine oxidase
MMAO	Mitochondrial matrix amine oxidase
Cu-AO	Copper-containing amine oxidases
TPQ	2,4,5-Trihydroxyphenylalanine quinone
$\Delta\Psi$	Membrane potential
MPT	Mitochondrial polyamine transporter
BUA	Butylamine
PUT	Putrescine
SPM	Spermine
SPD	Spermidine
S	Substrate
X	Mitochondria channel
J	Transport rate for polyamine
k_c	Catalytic constant for BSAO
K_t	Michaelis–Menten constant for transport
K	Generic equilibrium constant
ΔG	Gibbs free energy
ΔG^*	Activation Gibbs free energy
k_n	Uptake rate constant
R.C	Reaction coordinate
D.M.	Depth membrane
R	Gas constant
k_B	Boltzmann constant

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Introduction

Bovine serum (BSAO) and mitochondrial matrix (MMAO) amine oxidases are members of a heterogeneous class of enzymes-containing copper as cofactor and so denoted as *copper-containing amine oxidases* (Cu-AO) EC 1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper-containing)] (Boyce et al. 2009; Agostinelli et al. 2010). Amine oxidases are widely distributed in mammals, plants and microorganisms (prokaryotic and eukaryotic) (Pietrangeli et al. 2004; Medda et al. 2009; Okajima and Tanizawa 2009) being involved in polyamine metabolism and on many physio-pathological processes (Boyce et al. 2009). Despite their wide distribution, the physiological role of Cu-AOs is still unclear; however, in mammals Cu-AOs activity appears to be altered in some pathological conditions (Boomsma et al. 2009).

In general, they are glycosylated homodimers of subunit size of 70–95 kDa, depending on the source. In addition to a copper ion, each monomer contains one quinone cofactor that has been identified as 2,4,5-trihydroxyphenylalanine quinone (TPQ) (Janes et al. 1990).

Amine oxidases catalyze the oxidative deamination of primary amines producing the corresponding aldehyde, ammonia and hydrogen peroxide, according to the following scheme of reaction:

$$R - CH_2 - NH_2 + O_2 + H_2O \rightarrow R - CHO + NH_3 + H_2O_2$$

In the case of BSAO and MMAO, it has been reported that electrostatic interactions play a fundamental role in the polyamines–enzyme complex formation (Stevanato et al. 1994; Di Paolo et al. 2003), but the different specificity toward (poly)amine molecules of amine oxidases purified from different sources (Vianello et al. 1993, 1999; Di Paolo et al. 1995) suggests that this is a general behavior for this class of enzymes.

The naturally occurring polyamines, spermine, spermidine and putrescine, which are preferential substrates of the amine oxidases, are transported into the matrix space of mitochondria by a specific, common uniporter system. This transport process is dependent on the membrane potential ($\Delta\Psi$), thus demonstrating an electrophoretic behavior, and exhibits a non-ohmic flux–voltage relationship. In other words, polyamine transport happens by electrostatic interactions and the transport rate increases with increasing charge number of the transported species with an activation enthalpy of about 12 kJ/mol per charge at $\Delta\Psi \simeq 175$ mV (Toninello et al. 1988, 1992). Force-flux analyses provide evidences that the polyamine transporter is a suitable channel having two asymmetrical energy barriers with an energy well, in which is located the binding site, near the membrane surface, with spermine bound to the site in rapid equilibrium with the external spermine (Toninello et al. 2000).

These results obtained in independent manner, from functionally different protein structures and by different analytical and kinetic methods, show that in both cases electrostatic interactions control the protein functionality toward the same substrates.

The aim of this work is to compare experimental data in order to obtain further information on possible common mechanisms of oxidative deamination and transport process of these polyamines functionally dependent proteins.

Materials and methods

All chemicals were reagent grade and used without further purification.

Bovine serum and mitochondrial matrix amine oxidases were purified following the procedures of Vianello and Cardillo, respectively (Vianello et al. 1992; Cardillo et al. 2009). Protein concentration was measured according to the Bradford method, using bovine serum albumin as a standard.

Activity parameters by initial rate measurements were carried out spectrophotometrically, using the peroxidase-coupling assay previously reported (Stevanato et al. 1994).

Rat liver mitochondria were isolated as previously reported (Toninello et al. 1992) and the mitochondrial protein concentration was assayed by the biuret method with bovine serum albumin as a standard. Kinetic parameters of the transporter were obtained measuring the uptake of labeled polyamines ([^{14}C]spermine, [^{14}C]spermidine, [^{14}C]putrescine, 50 μC/mmol) by a centrifugal filtration method as previously described (Toninello et al. 1988).

Results

Polyamine transport across the mitochondrial membrane is a saturable process and the data of polyamine uptake rate can be treated following the theory of simple enzyme kinetics, according to the scheme of reaction:

$$X + S_o \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} XS \xrightarrow{k_2} X + S_i$$

where S_o is the polyamine at the outer side of the membrane, S_i is that at the inner side, X is a binding site in the channel and XS is the polyamine-binding complex. k_n represents the uptake rate constant of the n step.

Consequently, the experimental data of transport rate J can be treated according the Michaelis–Menten equation:

$$J = J_{max}[S]/(K_t + [S])$$

where J_{max} is the maximum rate at $[S] \gg K_t$, and K_t corresponds to the Michaelis–Menten constant.

Table 1 Kinetic parameters of polyamines transport by a mitochondria channel and oxidative deamination by amine oxidases from bovine serum and rat liver mitochondria matrix, respectively

Amine	Amine charge	MPT			BSAO			MMAO		
		J_{max} (μM/mg prot min)	K_t (mM)	J_{max}/K_t [1/mg prot min ($\times 10^{-3}$)]	k_{cat} (1/min)	K_m (mM)	k_{cat}/K_m [1/min ($\times 10^{+4}$)]	V_{max} [M/mg prot min ($\times 10^{-5}$)]	K_m (mM)	V_{max}/K_m [1/mg prot min ($\times 10^{-1}$)]
BUA	1.00				56	2.40	2.33			
PUT	1.99	1.14	1.00	1.14				4.00	0.749	0.534
SPD	2.98	1.23	0.26	4.73	114	0.28	40.7	6.80	0.108	6.30
SPM	3.86	1.41	0.13	10.8	139	0.076	183	10.9	0.023	47.4

All measurements were run in triplicate and SD values for J_{max} and V_{max} were ≤13%, while SD values for K_t and K_m were ≤19%

MPT mitochondrial polyamine transporter, *BSAO* bovine serum amine oxidase, *MMAO* mitochondrial matrix amine oxidase

Table 1 reports the kinetic parameters referred to (1) the polyamines transport by a mitochondrial polyamine transporter (MPT), and (2) the oxidative deamination of polyamines by two amine oxidases purified from bovine serum (BSAO) and rat liver mitochondria matrix (MMAO), respectively.

Plotting the data related to the maximum transport rate (J_{max} for the polyamine transporter) and to the catalytic constants (k_c in the case of BSAO and V_{max} in the case of MMAO) versus substrate charge (Fig. 1), it clearly appears a slight linear dependence with a good linear correlation coefficient. In all the cases, the transport rate and the catalytic constant values decrease in function of the positive charge from 4 to 1, corresponding to percentage values variable from about 20 to 60%, depending on the specific protein.

In contrast, the plot of the Michaelis–Menten constant values (K_t in the case of the mitochondria channel, K_m for the amine oxidases) versus substrate charge shows identical exponential behavior (Fig. 2a). Michaelis–Menten constant values increase of about 25–30 times decreasing the positive charge of substrate from 4 to 1. The logarithmic form plot of the experimental data (Fig. 2b) selects three straight lines with a good linear correlation coefficient characterized by slope values of −1.1, −1.2 and −1.9 for MPT, BSAO and MMAO, respectively. The intercepts on the *Y*-axis determine the Michaelis–Menten constant values for a hypothetical neutral substrate: 8.2 mM for the MPT and BSAO and 30 mM for MMAO.

Similarly, the plots of the natural logarithm of the maximum rates on Michaelis–Menten constants values versus substrate charge (Fig. 3) show straight lines, so indicating that the slight linear dependence of the catalytic constant with substrate charge does not affect the behaviors of the process at the first-order conditions, i.e. when the polyamine concentration values are much more lower than K_m or K_t.

From the Michaelis–Menten equation, $K_m = (k_c + k_{-1})/k_1$, if $k_c \ll k_{-1}$, the Michaelis constant is $K_m = k_{-1}/k_1 = K_s$, i.e. the dissociation constant of the protein–polyamine complex into the two components protein and polyamine. In this case, the contribution of a polyamine's single charge to the Gibbs free energy of the complex polyamine–protein formation can be determined.

In fact, $\Delta G = -RT \ln K$, from which

$$\Delta\Delta G = RT[\ln K_{n-1} - \ln K_n]$$

where the suffix n indicates the positive charge number of the polyamines.

In Table 2 line (a), these data for the three polyamine–protein complex taken into consideration are reported.

In contrast, if $k_c \gg k_{-1}$, $k_c/K_m = k_1$, i.e. the ratio between the catalytic constant or maximum transport rate and the Michaelis–Menten constant corresponds to the rate constant of formation of the complex protein–polyamine.

Applying the Eyring equation, $k = (k_B T/h)e^{-(\Delta G^*/RT)}$, where ΔG^* is the activation Gibbs free energy for the complex polyamine–protein formation, and developing we obtain

$$\Delta\Delta G^* = RT[\ln k_{n-1} - \ln k_n].$$

In Table 2 line (b), the contribution of each positive charge to the activation Gibbs free energy for the three complexes polyamine–protein formation taken into consideration are reported.

These values are similar to those reported by Gerstner et al. (1994) related to the Gibbs free energy of adsorption of several amino acids, peptides and proteins in a cation-exchange system and comparable to the bond energies for lysozyme toward a charged surface (Roth and Lenhoff 1993).

In other words, in the first hypothesis, i.e. for $k_c \ll k_{-1}$, experimental data indicate that the increase of a positive

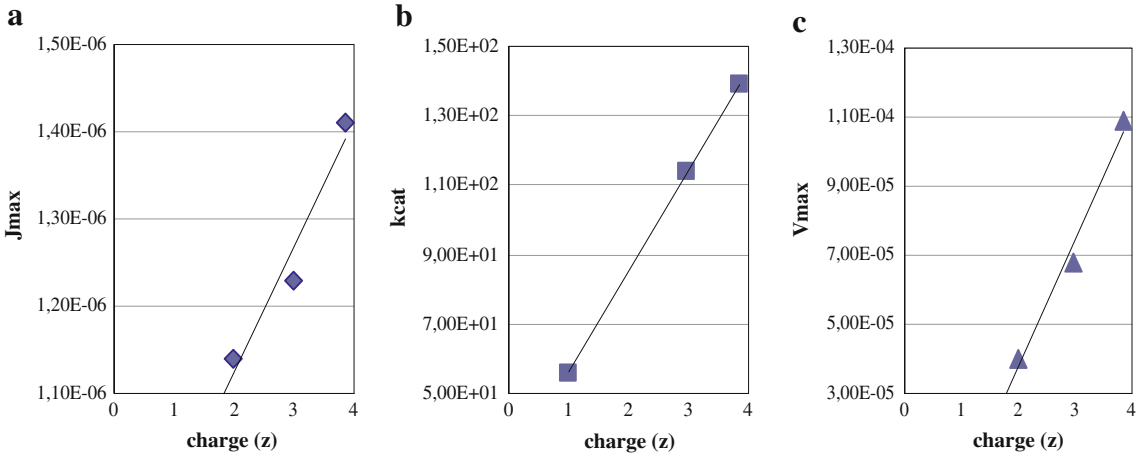
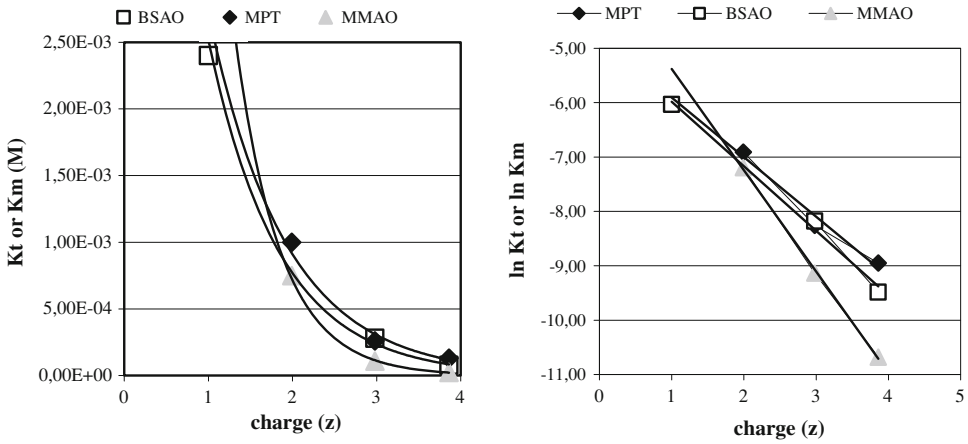


Fig. 1 Dependence on the substrate positive charge of **a** maximum transport rate for the polyamine transport (J_{max}); **b** catalytic constant for BSAO (k_{cat}) and maximum rate for MMAO (V_{max}). All measurements were run in triplicate and SD values for J_{max} and V_{max} were $\leq$13%

Fig. 2 **a** Dependence of Michaelis–Menten constants (K_m for amine oxidases and K_t in the case of mitochondria channel) on the substrate positive charge; **b** logarithmic form graph. All measurements were run in triplicate and SD values for K_t and K_m were $\leq$19%



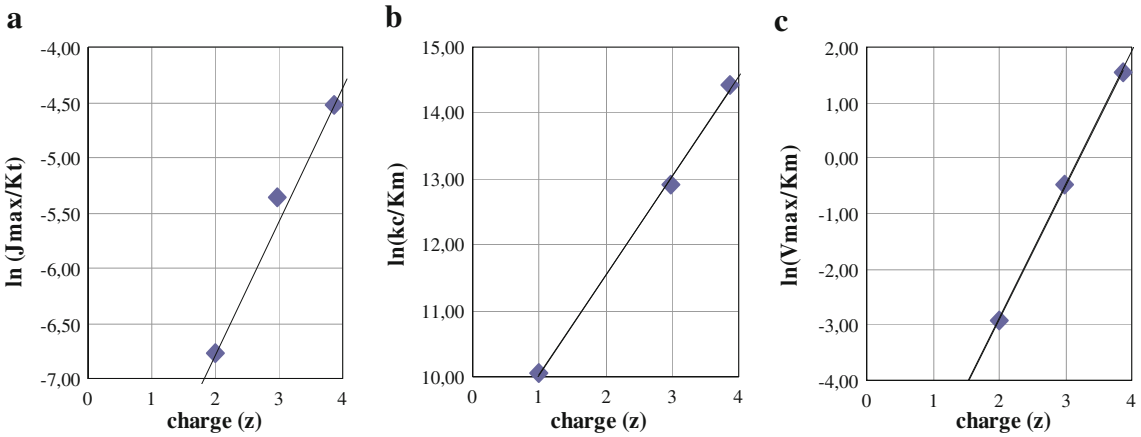


Fig. 3 Dependence of the natural logarithm of the ratio of maximum rates on Michaelis constants versus substrate charge for **a** MPT, **b** BSAO and **c** MMAO

charge in the polyamine substrate causes an increase of the Gibbs energy of the equilibrium constant relative to the polyamine–protein complex dissociation (or formation); i.e. the increase of a positive charge in the polyamine molecule stabilizes the polyamine–protein complex of a Gibbs energy value comparable to that of a single-charge electrostatic interaction due to the absorption of proteins into a cation-exchange system.

Table 2 Contribute of a positive charge of the polyamine to: (a) the Gibbs free energy of the complex polyamine–protein formation and (b) the activation Gibbs free energy for the complex polyamine–protein formation

Hypotheses		$\Delta\Delta G$	$\Delta\Delta G$ (kJ/mol)		
			MPT	BSAO	MMAO
(a)	$k_c \ll k_{-1}$	RT [ln K_{n-1} – ln K_n]	2.74	2.96	4.65
(b)	$k_c \gg k_{-1}$	RT [ln k_{n-1} – ln k_n]	−2.94	−3.68	−5.88

In the second case, i.e. for $k_c \gg k_{-1}$, the results indicate that the increase of a positive charge in the polyamine substrate decrease the activation energy of polyamine–protein complex of a Gibbs energy value comparable to that above cited. These differences of Gibbs energy are visualized in Fig. 4.

Discussion

Previous papers (Di Paolo et al. 2003; Stevanato et al. 1994, 2011) reported that two negative functional groups in the active site of bovine serum and mitochondrial matrix amine oxidases interact electrostatically with three positive charges of the polyamines in the formation of the enzyme–substrate complex. In the present paper we report that in both the cases, while the catalytic constant values show little changes with the number of the positive charges of the substrates, Michaelis–Menten constant values evidence strong exponential dependence, so confirming that electrostatic forces control the docking of the substrate into the enzyme active site.

By the treatment of the kinetic data in terms of Gibbs equation or Eyring theory, the contribution of each positive charge of the polyamine to the Gibbs energy values for the oxidative deamination of polyamines by two mammalian amine oxidases are obtained.

The treatment of the experimental values of J_{max} and K_t referred to the MPT, obtained according to the Michaelis–Menten theory applied to ion transport across leaks and channels, evidences a behavior very similar to that found for the two amine oxidases. The values of the Gibbs free energy decreasing, due to one positive charge increasing in the polyamine molecule, were comparable, compatibly with the different experimental conditions of measurement, in both cases: $k_c \ll k_{-1}$ and $k_c \gg k_{-1}$. Furthermore, these values were in good accordance with those reported in literature.

Remembering the structure–function relationship of proteins, these results suggest the hypothesis that also in the polyamine transporter two negatively charged groups must interact with the positive charges of the polyamines. In this hypothesis, the interacting groups must be sterically located in such a way as to maximize the electrostatic

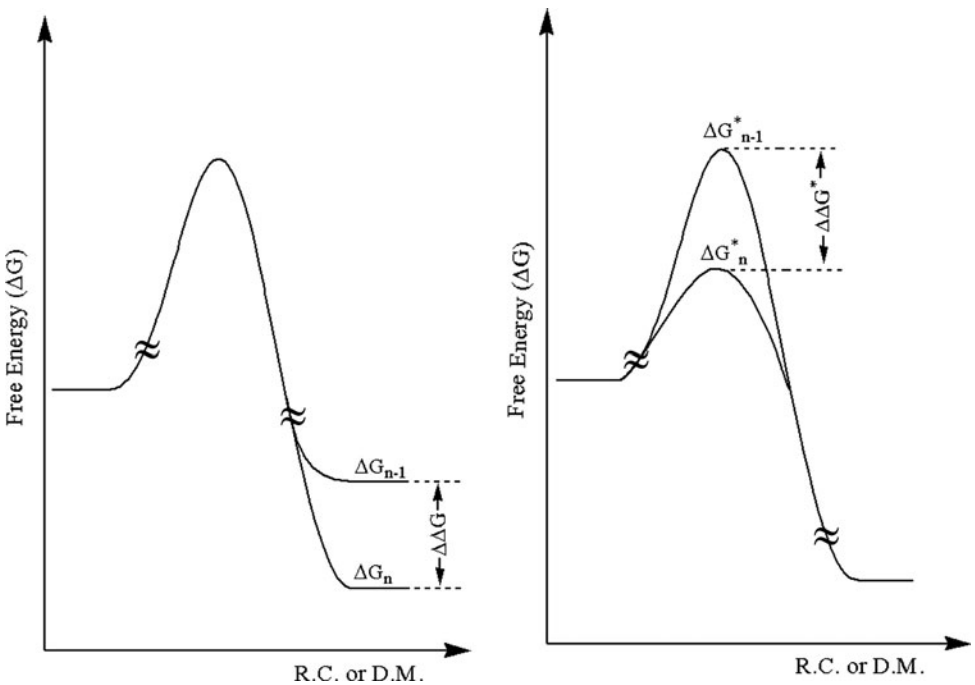


Fig. 4 Contribute of one positive charge of the polyamine to the Gibbs free energy, **a** of the complex polyamine–protein formation (in the case $k_c \ll k_{-1}$) and **b** of the activation complex for the complex polyamine–protein formation (for $k_c \gg k_{-1}$). *R.C* and *D.M.* indicate reaction coordinate or depth in membrane, respectively

interactions with the well determinate positions of the positive charges of the substrate such as in the amine oxidases active site it happens.

In particular, in the case of BSAO, two negatively charged aspartic residues interact electrostatically with the first and the third amino group of spermine or spermidine in the docking of the substrate into the active site. The aspartic residue farthest from the TPQ cofactor, interacting with the third amino residue, is located at the end of a flexible hairpin of a second subunit of the enzyme and is characterized by a high mobility that permits the docking of different substrates characterized by different chain length and charge distribution. This structure is substantially conserved in at least other five structurally characterized amine oxidases, although the dissimilarities in the architecture and charge distribution of the cavities leading to the active site could explain the differences in substrate specificity (Lunelli et al. 2005). The results here reported, suggest that a similar mechanism could regulate the introduction of the polyamines in the mitochondrial channel. In this hypothesis, a similar structural topology must be invoked, and, as a consequence, also the primary sequence of the polyamine-binding domains of the amine oxidases and the transporter may be conserved.

Recently, four complete genes were identified and characterized in the pig genome, each encoding amine oxidase copper-containing with different roles (diamine oxidase, retina-specific amine oxidase, vascular adhesion protein-1, serum amine oxidase), where two of these are membrane-associated proteins (Schwelberger 2010). By virtue of the identical exon–intron organization and of the degree of sequence conservation, a common evolutionary origin for the four genes has been hypothesized. These results further support the hypothesis of a high degree of sequence conservation in the proteins acting on polyamines, in particular in the step of polyamine docking. This structure could originate from an ancestral protein polyamine-recognizing gene.

In order to give consistence to this stimulating hypothesis, further experiments on mitochondria purified from bovine liver are in progress.

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References

Agostinelli E, Tempera G, Viceconte N, Saccoccio S, Battaglia V, Grancara S, Toninello A, Stevanato R (2010) Potential anticancer application of polyamine oxidation products formed by amine oxidase: a new therapeutic approach. Amino Acids 38:353–368

Boomsma F, van der Meiracker AH, Toninello A (2009) Plasma amine oxidases in various clinical conditions and in apoptosis. In: Floris G, Mondovì B (eds) Copper amine oxidases. CRC Press, Boca Raton, pp 143–158

Boyce S, Tipton KF, O'Sullivan MI, Davey GP, Gildea MM, McDonald AG, Olivieri A, O'Sullivan J (2009) Nomenclature and potential function of copper amine oxidases. In: Floris G, Mondovì B (eds) Copper amine oxidases. CRC Press, Boca Raton, pp 5–17

Cardillo S, De Iuliis A, Battaglia V, Toninello A, Stevanato R, Vianello F (2009) Novel copper amine oxidase activity from rat liver mitochondria matrix. Arch Biochem Biophys 485:97–101

Di Paolo ML, Vianello F, Stevanato R, Rigo A (1995) Kinetic characterization of soybean seedling amine oxidase. Arch Biochem Biophys 323:329–334

Di Paolo ML, Stevanato R, Corazza A, Vianello F, Lunelli L, Scarpa M, Rigo A (2003) Electrostatic versus hydrophobic interactions between bovine serum amine oxidase and its substrates. Biochem J 371:549–556

Gerstner JA, Bell JA, Cramer SM (1994) Gibbs free energy of adsorption for biomolecules in ion-exchange systems. Biophys Chem 52:97–106

Janes SM, Mu D, Wemmer D, Smith AJ, Kaur S, Maltby D, Burlingame AL, Klinman JP (1990) A new redox cofactor in eukaryotic enzymes: 6-hydroxydopa at the active site of bovine serum amine oxidase. Science 248:981–987

Lunelli M, Di Paolo ML, Biadene M, Calderone V, Battistutta R, Scarpa M, Rigo A, Zanotti G (2005) Crystal structure of amine oxidase from bovine serum. J Mol Biol 346:991–1004

Medda R, Bellelli A, Pec P, Federico R, Cona A, Floris G (2009) Copper amine oxidases from plants. In: Floris G, Mondovì B (eds) Copper amine oxidases. CRC Press, Boca Raton, pp 39–50

Okajima T, Tanizawa K (2009) Mechanism of TPQ biogenesis in prokaryotic copper amine oxidase. In: Floris G, Mondovì B (eds) Copper amine oxidases. CRC Press, Boca Raton, pp 103–118

Pietrangeli P, Morpurgo L, Mondovì B, Di Paolo ML, Rigo A (2004) Soluble copper amine oxidases from mammals. In: Floris G, Mondovì B (eds) Copper amine oxidases. CRC Press, Boca Raton, pp 51–68

Roth CM, Lenhoff AM (1993) Electrostatic and Van der Waals contributions to protein adsorption: computation of equilibrium constants. Langmuir 9:962–972

Schwelberger HG (2010) Structural organization of mammalian copper-containing amine oxidase genes. Inflamm Res 59:223–225

Stevanato R, Mondovì B, Befani O, Scarpa M, Rigo A (1994) Electrostatic control of oxidative deamination catalysed by bovine serum amine oxidase. Biochem J 299:317–320

Stevanato R, Cardillo S, Braga M, De Iuliis A, Battaglia V, Toninello A, Agostinelli E, Vianello F (2011) Preliminary kinetic characterization of a copper amine oxidase from rat liver mitochondria matrix. Amino Acids 40:713–720

Toninello A, Miotto G, Siliprandi D, Siliprandi N, Garlid KD (1988) On the mechanism of spermine transport in liver mitochondria. J Biol Chem 263:19407–19411

Toninello A, Dalla Via L, Siliprandi D, Garlid KD (1992) Evidence that spermine, spermidine and putrescine are transported electrophoretically in mitochondria by a specific polyamine uniporter. J Biol Chem 267:18393–18397

Toninello A, Dalla Via L, Stevanato R, Yagisawa S (2000) Kinetics and free Energy profiles of spermine tran sport in liver mitochondria. Biochemistry 39:324–331

Vianello F, Di Paolo ML, Stevanato R, Rigo A (1992) Isolation of amine oxidase from bovine plasma by a two-step procedures. Protein Expr Purif 3:362–367

Vianello F, Di Paolo ML, Stevanato R, Gasparini R, Rigo A (1993) Purification and characterization of amine oxidase from soybean seedlings. Arch Biochem Biophys 307:35–39

Vianello F, Malek-Mirzayans A, Di Paolo ML, Stevanato R, Rigo A (1999) Purification and characterization of amino oxidase from pea seedlings. Protein Expr Purif 15:196–201