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ORIGINAL ARTICLE

Preliminary kinetic characterization of a copper amine oxidase from rat liver mitochondria matrix

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Abstract Kinetic measurements of a novel copper-dependent amine oxidase, purified from rat liver mitochondria matrix, were carried out using various substrates in a large pH (5.6–10.2) and ionic strength range (5–200 mM), in order to study the docking of substrates to the enzyme and, as a consequence, to verify the physicochemical characteristics of the active site. Relatively small changes of V_{max} values (approx. 2.5-folds) over the substrates tested, suggest that the rate determining step of the catalysis is only slightly affected by amine chemical structure. In contrast, the strong change of K_M and k_c/K_M values (approx. two orders of magnitude) indicates electrostatic control of the docking process, since the changes of K_M and k_c/K_M values appear due to the presence of positively charged groups in the substrate molecules. These results suggest the presence in the enzyme active site of two negatively charged amino acid residues which seem to interact with positively charged groups of the substrate molecules. Analogies and differences with bovine serum amine oxidase are also described.

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Keywords Amine oxidases · Mitochondrion · Enzyme function · Ionic strength · Polyamines

Abbreviations

Cu-AO	Copper containing amine oxidases
BSAO	Bovine serum amine oxidase
MMAO	Mitochondrial matrix amine oxidase
SPM	Spermine
SPD	Spermidine
TPQ	2,4,5–Trihydroxyphenylalaninequinone
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
MES	2-Morpholinoethanesulfonic acid
MOPS	3-[*N*-Morpholino]propanesulfonic acid)
HEPPS	3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid
PUT	1,4-Diaminobutane (putrescine)
DIOXA	4,9-Dioxa-1,12-diaminododecane

Introduction

Copper containing amine oxidases (Cu-AO) represent a heterogeneous class of enzymes classified as EC 1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper-containing)], widely distributed in mammals, plants and microorganisms (prokaryotic and eukaryotic) (Knowles and Dooley 1994; Agostinelli et al. 2010). There is an increasing interest on this class of enzymes as they are involved in polyamine metabolism and on many physio-pathological processes (Petterson 1985; Agostinelli et al. 2004, 2007). Many Cu-AOs have been purified to homogeneity and characterized. In general, they are glycosylated homodimers

of subunit size of 70–95 kDa, depending on the source. Each monomer contains one Cu(II) and one quinone cofactor that has been identified as 2,4,5-trihydroxyphenylalanine quinine (TPQ) (Klinman and Mu 1994).

The oxidation of aromatic and aliphatic primary amines by Cu-AOs produces the corresponding aldehyde, ammonia and hydrogen peroxide, according to the following equation:

$$R-CH_2-NH_3^+ + O_2 + H_2O \xrightarrow{\text{Amine Oxidase}} R-CHO + NH_4^+ + H_2O_2 \quad (1)$$

Despite their wide distribution, the physiological role of Cu-AOs is still unclear. Anyway, in mammals Cu-AOs activity appears to be altered in some pathological conditions (Luhova et al. 1996; Rinaldi et al. 1985).

In the last years many kinetic studies were carried out (Farnun et al. 1986; Hartmann et al. 1993; Palcic and Klinman 1983; Bellelli et al. 1991; Medda et al. 1995; Su and Klinman 1998; Bellelli et al. 2000; Bisby et al. 2000; de Vries et al. 2000) and provided an excellent foundation for understanding the chemical steps of the catalytic mechanism, which are the steps involved in the breaking of C–H and C–N bonds of the substrate, and the insertion of an oxygen atom. In contrast, only few reports have been focused on the factors involved in the recognition process between the substrates and the active site of AOs (Bardsley et al. 1970; Bardsley and Ashford 1972; Hartmann and Klinman 1991; Equi et al. 1992; Stevanato et al.1994; Di Paolo et al. 1995, 2003; Luhova et al. 1996). In particular, in the case of bovine serum amine oxidase (BSAO), one of the most studied amine oxidases, an isotopic effect on k_c and on k_c/K_M values was observed using benzylamine, which is a poor, non-physiological substrate of this enzyme. This effect indicates the cleavage of the C–H bond as partially rate determining step of the catalytic process (Palcic and Klinman 1983; Hartmann et al. 1993; Klinman and Mu 1994). However, a relatively small change of the k_c values (less than a factor of three) (Palcic and Klinman 1983; Hartmann and Klinman 1991; Stevanato et al. 1994) and a strong change of K_M (over three orders of magnitude) were observed using various physiological substrates of BSAO. This behavior strongly supports the importance of physical interactions in the binding of the substrate to the enzyme since the changes of K_M values appear due to positively charged groups within the substrate molecule, not involved in the chemical step. In particular, the time course of, at least, BSAO-catalyzed processes appear controlled by physical rather than chemical factors (Di Paolo et al. 2003). There are many other examples in this field and one of these is the copper–zinc superoxide dismutase, which acts on the superoxide ion at a rate controlled by electrostatic interactions and not by the electron transfer process between the copper ion and superoxide (Argese et al. 1987).

In a previous paper we reported on the purification of a novel copper-dependent amine oxidase from rat liver mitochondrial matrix (Cardillo et al. 2009). The aim of the present work is the physico-chemical characterization of the active site of this new enzyme in order to verify similarity and the differences with BSAO, another mammalian copper containing enzyme, which shows high activity toward the same natural substrates, i.e. spermine and spermidine (Di Paolo et al. 2003; Agostinelli et al. 2010).

Materials and methods

All chemicals were of the highest available quality and were used without further purification. In particular, substrates used in this work were from Sigma–Aldrich (St. Louis, MO, USA). Spectrophotometric measurements were carried out with a Varian Cary 50 instrument.

Purification of amine oxidase in the soluble mitochondrial fraction

Amine oxidase from rat liver mitochondria matrix (MMAO) was purified as previously reported (Cardillo et al. 2009).

Enzyme activity measurements

Activity measurements were carried out according to a peroxidase-coupled assay (Stevanato et al. 1990) to continuously monitor the hydrogen peroxide produced during the oxidation of polyamines used as substrates.

Enzyme stability as a function of pH was determined in Britton–Robinson buffer, prepared by mixing 0.1 M boric acid, 0.1 M acetic acid and 0.1 M phosphoric acid. KOH (1 M) was added to achieve the desired pH.

In order to test whether variations of enzyme activity as a function of pH could be ascribed to inactivation or denaturation of MMAO during the measurements, the enzyme was incubated, at room temperature, in 33 mM Britton–Robinson buffer, at various pH in the range 5.0–8.5. Aliquots of protein solutions were withdrawn at various times and their activity was measured at pH 7.0 in the presence of 1 mM spermine and the half time of the enzyme was calculated.

Unless otherwise specified, all experiments were carried out in air-saturated solutions at room temperature (37 ± 1°C) in the presence of 0.1 mM EDTA. Enzyme

activity measurements as a function of ionic strength were carried out by addition of concentrated solutions of NaCl, KCl, $NaClO_4$ and $KClO_4$.

Data analysis

First-order (V_{max}) and second-order (V_{max}/K_M) rate constants were calculated from the V_{max} and K_M values, obtained by non-linear best-fit of experimental data on Michaelis–Menton equation, by assuming a MMAO molecular mass of 130 kDa (Cardillo et al. 2009).

Experimental data were fitted using the Sigma Plot 3.0 program (Jandel Scientific, San Rafael, CA, USA).

Results

Stability of mitochondrial matrix amine oxidase (MMAO) as a function of pH

In Fig. 1, where room temperature stability of MMAO as a function of pH is reported, it results that the enzyme appears to lose stability in the function of decreasing of the pH. In particular, the enzyme is relatively stable at pH values above 7.0 ($t_{1/2} \approx 13.5$ days), while at lower pH values a slow exponential decay was observed. In the pH range 6.0–5.0, the $t_{1/2}$ of the enzyme drops by two orders of magnitude and at pH 5.5 the calculated $t_{1/2}$ was about 21 h, considering that activity measurements were conducted within 5 min, the decay of enzyme activity due to enzyme inactivation was considered negligible at pH values above 5.5. Due to the rapid enzyme inactivation, activity measurements were not carried out at pH values below 5.5.

Dependence of activity of MMAO on pH

The dependences of activity parameters, V_{max}, K_M and V_{max}/K_M, of MMAO as a function of pH, in the pH range 5.6–10.2, were studied using spermine and putrescine as substrates.

Apparently, the experimental data referred to V_{max} do not describe a classical bell-shaped behavior (Fig. 2), according to the simplified scheme reported by Tipton and Dixon (Tipton and Dixon 1979) and verified in the case of BSAO (Di Paolo et al. 2003). As shown in Fig. 2, the behavior was similar for the two substrates investigated, a slightly decreasing trend increasing pH values, which does not permit the correct determination neither of the pH value of maximum activity, nor of the pK_a values controlling the pH dependence of V_{max}. In particular, for both substrates, a variation of V_{max} values less than 15% in the pH range 6.0–8.5 was observed, while a more marked decrease of the catalytic activity at pH values above 8.5 occurred.

Conversely, for the same substrates, K_M and, as a consequence, V_{max}/K_M values are strongly influenced by pH variations. The K_M values of MMAO toward the two substrates tend to increase in the whole pH range investigated. In the case of spermine, K_M extends from 2 μM at pH 6.0 to 300 μM at pH 9.5, while for putrescine K_M ranges from 60 μM at pH 6.0 to 1,100 μM at pH 9.5. In the

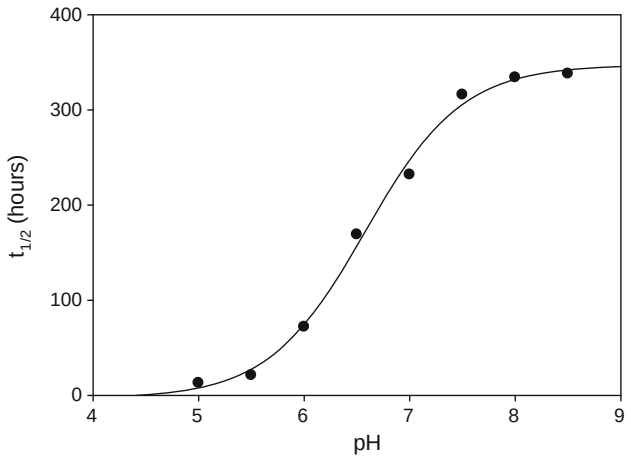


Fig. 1 Stability of MMAO as a function of pH. Aliquots of enzyme solution were incubated, at room temperature, in Britton and Robinson buffer at different pH values, then they were withdrawn at various times and their enzymatic activity was measured in 33 mM Britton–Robinson buffer equilibrated with air, at pH 5.0, in the presence of 0.1 mM spermine as substrate

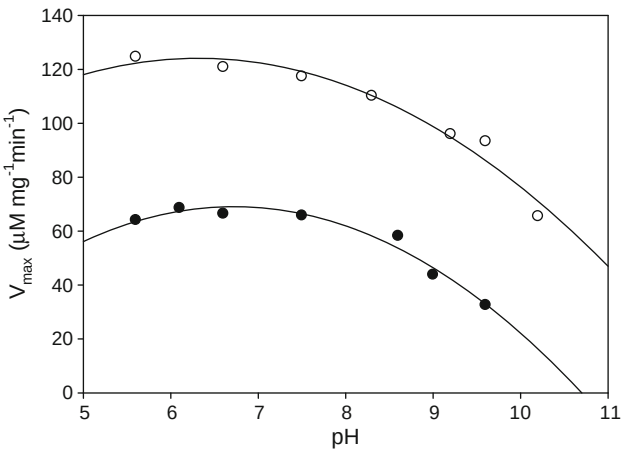


Fig. 2 Enzyme activity (V_{max}) as a function of pH using spermine and putrescine as substrates. Experiments were carried out with different buffers: MES in the pH range 5.6–6.1, MOPS at pH 6.6, HEPES at pH 7.5, HEPPS at pH 8.3, Borate in the pH range 8.6–9.2 and sodium carbonate in the pH range 9.6–10.2. Amine oxidase activity was spectrophotometrically determined as described in "Materials and methods". Symbols: *open circle* spermine, *closed circle* putrescine

logarithmic form (Fig. 3), a linear dependence of log(K_M) as a function of pH was observed ($R > 0.98$), characterized by a slope of +0.69 and +0.20 log(K_M)/pH unit for spermine and putrescine, respectively (Fig. 4).

The catalytic efficiency, V_{max}/K_M, of MMAO extends from 100 μM min^{-1} at pH 6.0 to 0.2 μM min^{-1} at pH 9.5, using spermine as substrate, while for putrescine V_{max}/K_M ranges from 0.2 μM min^{-1} at pH 6.0 to 0.01 μM min^{-1} at pH 9.5. For both the substrates considered, log(V_{max}/K_M) shows a linear decrease with pH ($R > 0.99$), the slopes being about −0.75 and −0.26 log(V_{max}/K_M)/pH unit for spermine and putrescine, respectively (Fig. 4).

All these results suggest the involvement of protonation–deprotonation processes in the recognition process between the enzyme active site and the substrate, but following a opposite trend with respect to BSAO; in fact in this last case, within the same pH range, the slopes of the graph reporting log(V_{max}/K_M) versus pH show positive values (Di Paolo et al. 2003).

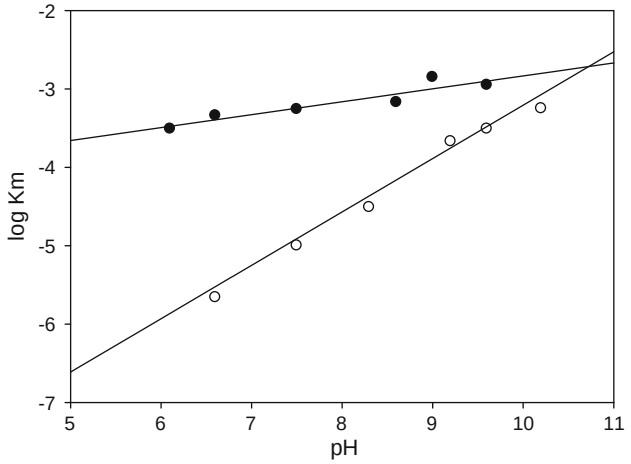


Fig. 3 Apparent enzyme affinity (expressed as log K_M) as a function of pH using spermine and putrescine as substrates. Experiments were carried out with different buffers: MES in the pH range 5.6–6.1, MOPS at pH 6.6, HEPES at pH 7.5, HEPPS at pH 8.3, Borate in the pH range 8.6–9.2 and sodium carbonate in the pH range 9.6–10.2. Amine oxidase activity was spectrophotometrically determined as described in "Materials and methods". Symbols: *open circle* spermine, *closed circle* putrescine

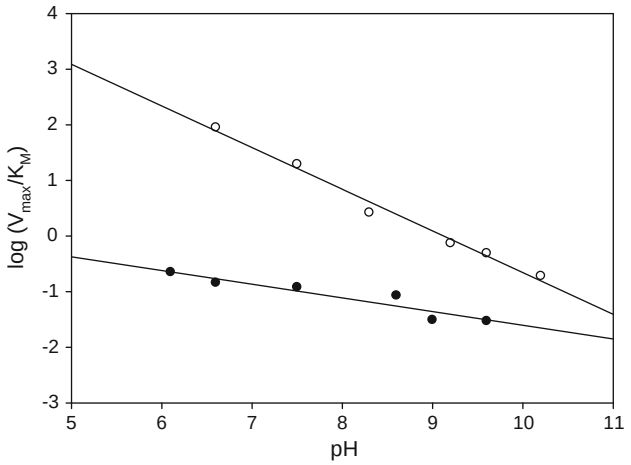


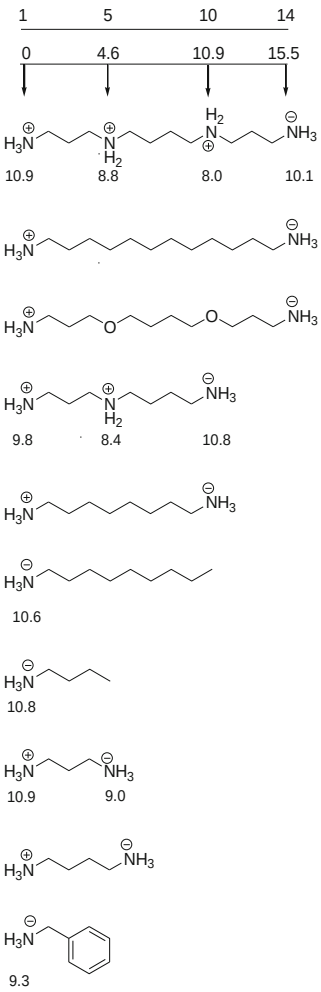
Fig. 4 Enzyme activity [expressed as log(V_{max}/K_M)]as a function of pH using spermine and putrescine as substrates. Experimental conditions were same as in Fig. 3. Symbols: *open circle* spermine, *closed circle* putrescine

Dependence of kinetic parameters on substrate structure

The dependence of MMAO activity on substrate structure has been studied using the amines reported in Table 1, where the kinetic parameters for each amine, from the initial rate measurements, are also reported.

Among the amines studied, it appears that the best fitted (the one presenting the lowest K_M and the higher V_{max} values) is spermine, followed by spermidine, as already reported for BSAO. Furthermore, K_M values of MMAO

Table 1 Structure and chain length of amines used as MMAO substrates



toward spermine and spermidine appear almost similar to those reported for BSAO (Di Paolo et al. 2003). From Table 2 it appears that V_{max} values of the analyzed substrates differ less than threefolds (from 37 to 109 μM min^{-1} mg^{-1}), indicating that the chemical steps controlling this parameter are relatively independent of the substrate structure. The higher values of V_{max} of SPM with respect to SPD could be due to the symmetry of the SPM molecule bearing two potentially reactive amino groups, instead of one, as in the case of SPD. In fact the amino-propyl end of SPD could be characterized by a much higher activity with respect to that of the amino-butyl end.

The Michaelis constant (K_M), and therefore the catalytic efficiency (V_{max}/K_M), appear sensibly dependent on the substrate structure. The V_{max}/K_M values increase approx. two magnitude orders from putrescine to spermine; on the other hand, diamines such as putrescine, 1,3-diaminopropane, 1,8-diaminooctane, 1,12-diaminododecane and 4,9-dioxa-1,12-diaminododecane show very similar values of V_{max} and K_M. Furthermore, the enzyme shows un-determinable catalytic parameters toward monoamines of short (butylamine, benzylamine) and long (*n*-nonylamine) aliphatic chain.

In brief, the activity data for the investigated amines can be grouped into four classes:

- spermine, which shows the highest catalytic constant and the lowest Michaelis constant and, as a consequence, the highest catalytic efficiency;
- spermidine, showing a catalytic efficiency approx. one order of magnitude lower than that of spermine;
- diamines, such as 1,3-diaminopropane, 1,4-diaminonobutane, 1,8-diaminooctane, 1,12-diaminododecane, 4,9-dioxa-1,12-diaminododecane, which show similar values of catalytic efficiency, approx. two orders of magnitude lower than that of spermine;
- monoamines, such as 1-aminobutane, benzylamine and 1-aminononane, which show an un-determinable activity independent of the aliphatic chain length.

Therefore, conversely with data reported for BSAO, MMAO shows an activity with respect to diamines but not towards monoamines, but similar to BSAO, MMAO also shows the favorable effect of positively charged groups of the substrate on the enzyme-substrate interaction.

Dependence of V_{max} and K_M on ionic strength

The dependence of the kinetic rate constants, V_{max} or V_{max}/K_M, on ionic strength (I) at pH 7.0, at 37° C, was studied using the same substrate reported in Table 1. Experiments were carried out, in the I range 10–200 mM, by addition in the assay mixture of concentrated solutions of NaCl, KCl, $NaClO_4$ and $KClO_4$. No effect of the nature of the salt used on enzyme activity was observed. The kinetic data were analyzed according to the Debye Hückel equation (Atkins, 1986):

$$\log k = \log k_0 + 2CZ_aZ_b I^{1/2} \quad (2)$$

where k is K_M, V_{max} or V_{max}/K_M, Z_a and Z_b are the charges of the species involved in the formation of the enzyme-substrate complex and k_0 is the value of the constant, k, at $I = 0$ and the constant C is:

$$C = 1.82 \times 10^6 \times \rho^{1/2}(\varepsilon T)^{-3/2} \quad (3)$$

where ε and ρ represent the dielectric constant and the density of the medium, respectively (Leidler and Bunting, 1973). The constant C, in water and at 37°C, is 0.523 $M^{1/2}$.

From the analysis of the kinetic data, obtained at various ionic strength values, it appears that the V_{max} values of the tested substrates are almost independent of ionic strength, while a strong dependence of K_M and V_{max}/K_M was found, see Table 3 where the slopes of the dependences of the kinetic parameters versus $I^{1/2}$ are reported.

In particular, a linear dependence of $\log(K_M)$ and $\log(V_{max}/K_M)$ on $I^{1/2}$ was found for all the substrate studied. From these plots, the value of $2CZ_aZ_b$ for each

Table 2 Kinetic parameters of MMAO substrates

Substrate	Kinetic parameters		
	K_M (μM)	V_{max} (μM min^{-1} mg^{-1})	V_{max}/K_M (min^{-1} mg^{-1})
Spermine	23 ± 16	109 ± 47	4.74
Spermidine	108 ± 23	68 ± 23	0.63
1,3-Diaminopropane	695 ± 266	37 ± 4	0.053
1,4-Diaminobutane (putrescine)	749 ± 197	40 ± 25	0.053
1,8-Diaminooctane	674 ± 216	55 ± 2	0.082
1,12-Diaminododecane	303 ± 41	41 ± 29	0.14
4,9-Dioxa-1,12-diaminododecane	636 ± 260	47 ± 6	0.074
1-Aminononane	n.d.	n.d.	n.d.
1-Aminobutane	n.d	n.d	n.d
Benzylamine	n.d.	n.d.	n.d.

The kinetic measurements were carried out in 20 mM Hepes, pH 7.0. For the other experimental conditions see "Materials and methods"

All the set of measurements were run in triplicate

n.d. not determinable

Table 3 Slopes ($2CZ_aZ_b$) of the plots of kinetic parameters versus $I^{1/2}$

Substrate	Slopes of the dependences of the kinetic parameters vs. $I^{1/2}$ ($2CZ_aZ_b$)		
	K_M	V_{max}	V_{max}/K_M
Spermine (SPM)	+6.3 ± 1.5	−0.6 ± 0.8	−6.0 ± 0.4
Spermidine (SPD)	+5.8 ± 1.0	−0.5 ± 0.3	−6.3 ± 0.7
1,4-diaminobutane (putrescine, PUT)	+4.2 ± 0.6	−0.1 ± 0.2	−3.9 ± 0.4
1,3-diaminopropane	+4.4 ± 0.4	−0.1 ± 0.3	−4.2 ± 0.1
1,8-diaminooctane	+3.7 ± 0.4	−0.7 ± 1.0	−4.2 ± 1.6
1,12-diaminododecane	+1.9 ± 0.6	+0.2 ± 0.1	−1.7 ± 0.6
4,9-dioxa-1,12-diaminododecane (DIOXA)	+1.6 ± 0.4	+0.2 ± 0.2	−1.7 ± 0.5

Amine oxidase activity was spectrophotometrically determined in according to Stevanato et al. (1990) in 1 ml 20 mM Hepes, pH 7.5, containing 3 mM *N*,*N*-dimethyl-aniline, 3 mM amino-phenazone, 0.4 μM horseradish peroxidase. Determination was carried out at 555 nm

Ionic strength was determined using different salts (NaCl, $NaClO_4$, KCl and $KClO_4$) in the concentration range 5–200 mM

substrate was calculated and reported in Table 3. The $2CZ_aZ_b$ values referred not only to V_{max}/K_M, but also to K_M, with the only difference of the change of sign, can be grouped into three classes:

- Spermine and spermidine: $2CZaZ_b \approx -6$;
- 1,3-diaminopropane, 1,4-diaminobutane, 1,8-diaminooctane: $2CZ_aZ_b \approx -4$;
- 1,12-diaminododecane, 4,9-dioxa-1,12-diaminododecane: $2CZ_aZ_b \approx -2$.

The substantially identical value of $2CZ_aZ_b \approx -4$ for the diamines, 1,3-diaminopropane, 1,4-diaminobutane, 1,8-diaminooctane, suggests the possibility of an interaction between two negative charges of the active site with two positive charges of substrate. This hypothesis fits well also for spermine and spermidine ($2CZ_aZ_b \approx -6$), where two negatively charged residues in the enzyme active site can interact with three positive charges of spermidine and spermine, considering in this last case that the amino group in position 14 does not interact with the positively charged pocket of the active site for distance reasons, as already found in the case of BSAO (Di Paolo et al. 2003). This considerations appear confirmed by the data obtained for 1,12-diaminododecane and 4,9-dioxa-1,12-diaminododecane, where the product of interacting charges is near to −2, indicating the possible interaction of two positive charges of the active site with the only one positively nitrogen group in position 1 of the substrate, being, also in this case, the nitrogen atom in position 14 too far from N1 site, and then not sensed by the enzyme positively charged active site.

Discussion

In the previous paper (Di Paolo et al. 2003) we reported a hypothetic BSAO active site model deduced by activity measurements under different experimental physico-chemical conditions and using, as substrates, amines structurally related to spermine and spermidine, having the highest affinity for the enzyme. The active site model proposed was later substantially confirmed by spectroscopic (Di Paolo et al. 2007) and X-ray diffraction investigations (Lunelli et al. 2005), thus demonstrating that information on chemical and structural active site characteristics can be correctly inferred from kinetic measurements.

With respect to BSAO, rat liver MMAO shows analogies and differences which suggest some structural diversities at the active site level (Table 4).

The V_{max} versus pH behavior of MMAO appears strongly different from that of BSAO: in the first case the highest activity is not precisely definable being confined at pH values ≤6, where enzyme stability is very poor. Furthermore, V_{max} values decrease very slowly at alkaline pH: using spermine as substrate, ≈78% residual activity at pH 9 was found, while in the case of BSAO residual activity at the same pH value was ≈13% of that determined at optimum pH. The V_{max} versus pH behavior of MMAO could be explained as a classical bell shaped curve as well, but it is characterized by a very large outline, not completely determinable as a consequence of the low enzyme stability at pH values <6 and >10. The hypothetic pK_a values of the protonated groups controlling the pH dependence of the catalytic constant can be indicatively positioned at pH values <6 and >8.5. These values appear substantially different from those found for BSAO (6.5 and 7.5, respectively). Furthermore, MMAO shows a negligible activity towards monoamines with short (1-aminobutane, benzylamine) or long (1-aminononane) aliphatic chains, suggesting the lack of the hydrophobic region found in the BSAO active site (Di Paolo et al. 2003). This hydrophobic region, confirmed by X-ray diffraction studies (Lunelli

Table 4 Main differences between MMAO and BSAO

Characteristic	MMAO	BSAO
Source	Liver mitochondrial matrix	Bovine serum
Molecular weight (kDa)	130	170[a]
Activity on monoamines	No	Yes
pK_a of the more acidic residue in the active site	<6.0	6.5
pK_a of the more basic residue in the active site	>8.5	7.5
Presence of a flexible chain ending with a carboxylic group	Yes	No
Presence of a hydrophobic pocket in the active site	No	Yes

[a] According to Bellelli et al. (2000)

et al. 2005) and characterized by a dielectric constant $\varepsilon_r \approx 10$ (Di Paolo et al. 2007) could justify the narrow V_{max} versus pH profile of BSAO in comparison with that found for MMAO. It is known, in fact, that acidic dissociation constants, K_a, decrease lowering the dielectric constant of the medium for acidic functional groups, but increase for basic functional groups. As verified for BSAO (Di Paolo et al. 2003), the two functional groups controlling the pH dependence of V_{max} could be the same in the two enzymes (carboxylic groups), but with very different pK_a values in relation to the different micro-environmental dielectric constants.

As already reported for BSAO (Di Paolo et al. 2003), the independence of V_{max} values on ionic strength in comparison to the strong dependence of V_{max}/K_M values suggests that the oxidative deamination of amines by MMAO occurs in two main steps: an initial step controlled by physical factors, which supports binding of the substrate to the enzyme active site, followed by a chemical step, during which the complex enzyme–substrate evolves to the end products. So, as for BSAO, also for MMAO, the chemical step appears relatively independent of the substrate structure since the values of the kinetic rate constants (V_{max}) controlling this step lye in a relatively narrow range (37–109 μM min^{-1} mg^{-1}).

With regards to physical step of the enzyme process, the strong dependence of K_M and V_{max}/K_M values (more than two magnitude orders) on the amine structure appears due to the presence of positively charged N atoms in position 1, 5, 10. In fact, the strong dependence of K_M and V_{max}/K_M on ionic strength suggests that substrate binding is controlled by electrostatic interactions between charges of opposite sign and, in particular, between positive amine groups of the substrate and negatively charged groups, indicatively, carboxylic groups of the enzyme. In the case of spermine, we must consider the interaction between the positively charged N atom in position 1, 5 and 10, i.e., the same amino groups of spermidine, and two negatively charged residue of the enzyme.

These considerations support a model of MMAO active site more simplified with respect to that reported for BSAO.

The main characteristics of this model can be briefly summarized as follows:

1. Two negatively charged carboxylic groups in the enzyme active site interact with positively charged amino groups of the substrate, positioned, in the case of spermine and spermidine, in N1, N5, N10.
2. Linear α-ω-diamines interact with the active site in the same manner, independent of the aliphatic chain length, indicating that at least one of the hypothesized interacting carboxylic groups into the active site is positioned at the end of a flexible chain, to adapt its position in relation to the chain length of the substrate. It is plausible to hypothesize that this characteristic concerns the carboxylic group more distant from the TPQ, being the one closest to TPQ aimed to accomplish the catalytic step. The gap between the two extreme positions is about 6.3 Å, ranging from 4.6 Å of the second amino group of 1,3-diaminopropane to the 10.9 Å of the second amino group of 1,8-diaminoctane.
3. According to this model, 1,12-diaminododecane and 4,9-dioxa-1,12-diaminododecane should not show catalytic activity, being their interactions into the active site similar to those of monoamines (only the first amino group should interact). We hypothesize that a second interaction could happen between the ω-amino group of these long diamines and a non-charged group belonging to the enzyme active site, localized about 15 Å apart from the reactive TPQ cofactor. This hypothesis would also justify the higher affinity toward the active site of spermine when compared with spermidine, as found in MMAO and in BSAO as well. The interaction could involve a hydrogen bond or a charge–dipole interaction, which is not dependent on ionic strength, the product being $2CZ_aZ_b = 0$.
4. The MMAO active site neither seem to contain the hydrophobic pocket described for BSAO (Di Paolo et al. 2007), nor the protonated group assuming an "on/off" role in the docking of substrates (Di Paolo et al. 2003).

About the physiological role of MMAO, it is to consider that in a previous paper, it was reported that the biogenic amine, agmatine, is oxidized in liver mitochondria by inducing an oxidative stress, responsible of the transition pore opening (Battaglia et al. 2007). This effect has been observed in this type of mitochondria (Battaglia et al. 2010) and has been attributed to the activity of MMAO (Cardillo et al. 2009). Taking into account that the phenomenon of pore opening is related to the activation of the pro-apoptotic pathway, it is possible to state that MMAO plays an important role in this process and, consequently, in protecting the liver against neoplastic proliferation.

Conflict of interest The authors declare that they have no conflict of interest.

References

Agostinelli E, Arancia G, Dalla Vedova L, Belli F, Marra M, Salvi M, Toninello A (2004) The biological functions of polyamine oxidation products by amine oxidases: perspectives of clinical applications. Amino Acids 27:347–358

Agostinelli E, Tempera G, Molinari A, Salvi M, Battaglia V, Toninello A, Arancia G (2007) The phisiological role of biogenic amines redox reactions in mitochondria. New perspective in cancer therapy. Amino Acids 33:175–187

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

Argese E, Viglino P, Rotilio G, Scarpa M, Rigo A (1987) Electrostatic control of the rate determining step of Cu, Zn superoxide dismutase catalytic reaction. Biochemistry 26:3324–3328

Atkins PW (1986) Physical chemistry 3rd edn. Oxford University Press, Oxford. pp 242–243

Bardsley WG, Ashford JS (1972) Inhibition of pig kidney diamine oxidase by substrate analogues. Biochem J 128:253–263

Bardsley WG, Hill CM, Lobley RW (1970) A reinvestigation of the substrate specificity of pig kidney diamine oxidase. Biochem J 117:169–176

Battaglia V, Rossi CA, Colombatto S, Grillo MA, Toninello A (2007) Different behavior of agmatine in liver mitochondria: inducer of oxidative stress or scavenger of reactive oxygen species? Biochim Biophys Acta 1768:1147–1153

Battaglia V, Grancara S, Mancon M, Cravanzola C, Colombatto S, Grillo MA, Tempera G, Agostinelli E, Toninello A (2010) Agmatine transport in brain mitochondria: a different mechanism from that in liver mitochondria. Amino Acids 38:423–430

Bellelli A, Finazzi-Agrò A, Floris G, Brunori M (1991) On the mechanism and rate of substrate oxidation by amine oxidase from lentil seedlings. J Biol Chem 26:20654–20657

Bellelli A, Morpurgo L, Mondovì B, Agostinelli E (2000) The oxidation and reduction reactions of bovine serum amine oxidase. A kinetic study. Eur J Biochem 267:3264–3269

Bisby RH, Johnson AS, Parker AW (2000) Radicals from one-electron oxidation of 4-aminoresorcinol: model of the active site radical intermediate in copper amine oxidase. J Phys Chem 104:5832–5839

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

De Vries S, van Spanning RJM, Steinebach V (2000) A spectroscopic and kinetic study of Escherichia coli amine oxidase. J Mol Cat B: Enzymatic 8:111–120

Di Paolo ML, Vianello F, Stevanato R, Rigo A (1995) Kinetic characterization of soybean seedling amine oxidase. Arch Biochem Biophys 323:329–333

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

Di Paolo ML, Pesce C, Lunelli M, Scarpa M, Rigo A (2007) N-alkanamines as substrates to probe the hydrophobic region of bovine serum amine oxidase active site: a kinetic and spectroscopic study. Arch Biochem Biophys 465:50–60

Equi MA, Cooper A, Robins DJ (1992) Chain-length dependence of oxidation of α,ω-diamines by diamine oxidase. J Chem Res 8:94–95

Farnun M, Palcic M, Klinman JP (1986) pH dependence of deuterium isotopic effects and tritium exchange in the bovine plasma amine oxidase reaction: a role for single base catalysis in amine oxidation and imine exchange. Biochemistry 25:1898–1904

Hartmann C, Klinman JP (1991) Structure-function studies of substrate oxidation by bovine serum amine oxidase: relationship to cofactor structure and mechanism. Biochemistry 30:4605–4611

Hartmann C, Brozovic P, Klinman JP (1993) Spectroscopic detection of chemical intermediates in the reaction of para-substituted benzylamine with bovine serum amine oxidase. Biochemistry 32:2234–2241

Klinman JP, Mu D (1994) Quinoenzymes in biology. Ann Rev Biochem 63:299–344

Knowles PF, Dooley DM (1994) Amine oxidases. In: Sigel H, Sigel A (eds) Metal ions in biological systems. Marcel Dekker, New York. 30:361-403

Leidler K, Bunting P (1973) The chemical kinetics of enzyme action, 2nd edn. Clarendon Press, Oxford. 52-53

Luhova L, Slavik L, Frebort I, Sebela M, Zajoncova L, Pec P (1996) Comparison of kinetic properties between plant and fungal amine oxidases. J Enzyme Inhib 10:251–262

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, Padiglia A, Pedersen JZ, Rotilio G, Finazzi-Agrò A, Floris G (1995) The reaction mechanism of copper amine oxidase: detection of intermediates by the use of substrates and inhibitors. Biochemistry 34:16375–16381

Palcic MM, Klinman JP (1983) Isotopic probes yield microscopic constants: separation of binding energy from catalytic efficiency in the bovine plasma amine oxidase reaction. Biochemistry 22:5957–5966

Petterson G (1985) Plasma amine oxidase. In: Mondovi B (ed) Structure and function of amine oxidases. CRC Press, Boca Raton, pp 105–120

Rinaldi A, Floris G, Giartosio A (1985) Plant amine oxidase. In: Mondovi B (ed) Structure and function of amine oxidases. CRC Press, Boca Raton, pp 51–62

Stevanato R, Mondovì B, Sabatini S, Rigo A (1990) Spectrophotometric assay for total polyamines by immobilized amine oxidases. Anal Chim Acta 237:391–397

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

Su Q, Klinman JP (1998) Probing the mechanism of proton coupled electron transfer to dioxygen: the oxidative half-reaction of bovine serum amine oxidase. Biochemistry 37:12514–12525

Tipton KF, Dixon HBF (1979) Effects of pH on enzymes. Methods Enzymol 63:183–233