Electrostatic compared with hydrophobic interactions between bovine serum amine oxidase and its substrates

Maria Luisa DI PAOLO*, Roberto STEVANATO†, Alessandra CORAZZA‡, Fabio VIANELLO*, Lorenzo LUNELLI§, Marina SCARPA§ and Adelio RIGO*[1]

*Dipartimento di Chimica Biologica, Università di Padova, Via G. Colombo 3, 35121 Padova, Italy, †Dipartimento di Chimica Fisica, Università di Venezia, 30100 Venezia, Italy, ‡Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, 33100 Udine, Italy, and §Dipartimento di Fisica and INFM, Università di Trento, Via Sommarive 14, 38050 Povo-Trento, Italy

A steady-state kinetic study of bovine serum amine oxidase activity was performed, over a wide range of pH values (5.4–10.2) and ionic strength (10–200 mM), using various (physiological and analogue) substrates as specific probes of the active-site binding region. Relatively small changes in k_{cat} values (approx. one order of magnitude) accompanied by marked changes in K_m and k_{cat}/K_m values (approx. six orders of magnitude) were observed. This behaviour was correlated with the presence of positively charged groups or apolar chains in the substrates. In particular, it was found that the docking of the physiological polyamines, i.e. spermidine and spermine, appears to be modulated by three amino acid residues of the active site, which we have named L^-H^+, G^-H^+ and IH^+, characterized by pK_a values of 6.2±0.2 [Di Paolo, Scarpa, Corazza, Stevanato and Rigo (2002) Biophys. J. **83**, 2231–2239], 8.2±0.3 and 7.8±0.4 respectively. The electrostatic interaction between the protonated substrates and the enzyme containing the residues L^-H^+, G^-H^+ and IH^+ in the deprotonated form, the on/off role of the IH^+ residue and the role of hydrophobic interactions with substrates characterized by apolar chains are discussed.

Key words: amine oxidase, electrostatic interactions, enzyme function, hydrophobic interactions, ionic strength, polyamine.

INTRODUCTION

The copper-containing amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper-containing); EC 1.4.3.6] are a class of ubiquitous enzymes that are involved in the cellular and extracellular metabolism of amines. These enzymes catalyse the two-electron oxidation of primary amines to the corresponding aldehyde (oxidative half-reaction), with the reduction of molecular oxygen to hydrogen peroxide (reductive half-reaction), according to the following overall reaction [1–3]:

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

The nature of the covalently bound cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ), as well as the role of copper and the reaction mechanism of amine oxidases, has been investigated over many years [4]. Many kinetic and spectroscopic studies [5–13] have been carried out in an attempt to understand the catalytic mechanism, and have focused on the steps of the reductive half-reaction and on the formation of reaction intermediates, which are now well established. Conversely, the factors involved in the recognition and interaction between the substrate and the active site of amine oxidases are not so well established, owing to: (i) the wide range of amine substrates (from mono- to poly-amines), (ii) the differing substrate specificity among amine oxidases isolated from different sources, and (iii) the remarkably broad specificity of some amine oxidases, such as bovine serum amine oxidase (BSAO). Notwithstanding, some information on the interactions that occur between substrate and enzyme, as well as some structural information on the enzyme active site, has been obtained by kinetic studies carried out with a variety of substrates and inhibitors [5,14–22]. Furthermore, analysis of the crystal structures of some amine oxidases [23] and of their site-directed mutants obtained in the presence of covalently bound inhibitors [24,25] has provided a good view of the active-site channel.

With regard to BSAO, the structure of which has not yet been resolved, the relatively small change in the k_{cat} value (lower than a factor of three) [16,17,19] and the substantial change in K_m (over three orders of magnitude) observed using various substrates strongly suggest the importance of physical interactions in substrate binding to the enzyme and, consequently, on its catalytic efficiency. In fact, according to the minimal scheme describing an enzyme process, i.e.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}S \xrightarrow{k_{cat}} E + P \quad (2)$$

the reaction rate (v) is given by the Michaelis–Menten equation, and information about the physical factors responsible for substrate docking (diffusion, electrostatic interactions, etc.) could be obtained from the ratio k_{cat}/K_m, in the case both of $k_{cat} \gg k_{-1}$ ($k_{cat}/K_m \cong k_1$) and of $k_{cat} \ll k_{-1}$ ($K_m \cong k_{-1}/k_1$).

In the present paper, we report on the docking of physiological polyamines and of related compounds to BSAO. A general insight into the experimental results presented was gained using information on the deposited three-dimensional structures of some copper amine oxidases.

EXPERIMENTAL

Materials

All chemicals were of the highest available quality, and were used without further purification. In particular, the substrates used were from Sigma-Aldrich S.r.l. (Milan, Italy). BSAO was purified according to the procedure reported by Vianello et al. [26], and

Abbreviations used: BSAO, bovine serum amine oxidase; ECAO, *Escherichia coli* amine oxidase; PSAO pea seedling amine oxidase; TPQ, 2,4,5-trihydroxyphenylalanine quinone.

[1] To whom correspondence should be addressed (e-mail adelio.rigo@unipd.it).

the specific activity was found 0.36 unit/mg. One enzyme unit corresponds to 1 μmol of substrate transformed/min. The concentration of the purified enzyme was determined according to the method of Bradford [27], assuming a molecular mass of 180 kDa.

Enzyme activity measurements

Initial-rate measurements were carried out using the peroxidase-coupled assay reported by Di Paolo et al. [28]. A Perkin–Elmer Lambda-17 spectrophotometer was used for these measurements.

All measurements were carried out at 37 °C, in air-equilibrated solutions, i.e. under saturation conditions for molecular oxygen (K_m for O_2 of 10–20 μM [19,29]). Under these conditions, the kinetic parameters that we obtained by activity measurements were those of the other substrate (the amines) (see eqn 1). Ionic strength was controlled by suitable addition of NaCl.

Activity measurements performed at various pH values were carried out in the appropriate buffer (25 mM) containing NaCl (150 mM). The following buffers were used: Mes (pH 5.6–6.6), Mops (pH 6.6–7.3), Hepes (pH 7.3–7.8), Hepp (pH 7.8–8.6), borate (pH 8.6–9.6) and carbonate (pH 9.6–10.5). NaOH and HCl were used to adjust the pH. Activity measurements performed at overlapping pH values showed no significant influence of type of buffer on k_{cat} or K_m values.

A standard assay was carried out at 37 °C, using a solution containing 0.5 mM spermine, 25 mM Hepes and 150 mM NaCl, at pH 7.20. To account for possible changes in enzyme activity, the standard assay was carried out along with each set of rate measurements. The ratio of the observed rate to the rate of 0.36 unit/mg (see above) provided a correction factor that was then applied to all measurements within a given set.

Kinetic analysis

The reciprocal of the initial rate of reaction was plotted against the reciprocal of substrate concentration, and the k_{cat}, K_m and k_{cat}/K_m values were calculated according to the Lineweaver–Burk method. The k_{cat} parameter is the number of molecules of substrate transformed/s per catalytic centre (two per BSAO molecule).

Experimental data were fitted to the appropriate equation by using the least-squares method and the Sigma Plot 5.0 program (Jandel Scientific, San Rafael, CA, U.S.A.). Unless stated otherwise, the correlation coefficient for linear regression was 0.98 or greater. In the case of non-linear regression analysis, the value of the parameter obtained from the best fit and its S.E.M. are reported. All experiments were performed in triplicate.

Structural analysis

The data for the resolved crystal structures of amine oxidases from *Escherichia coli* (ECAO) and from pea seedlings (PSAO) were downloaded from the Protein Data Bank (PD ID: 1OAC and 1KSI respectively).

Hydrogen atoms were added to the molecule using the Builder module of the program Insight II (Molecular Simulation Inc., San Diego, CA, U.S.A.). Partial charges were assigned according to the AMBER force field [30]. In order to reduce computational time and memory storage, only a subset of residues of the protein, centred on the active site, was considered. These residues that form and surround the active site channel were located within volumes of approx. 35 Å × 35 Å × 70 Å and 30 Å × 30 Å × 55 Å for PSAO and ECAO respectively. The ionization state of the residues used in the simulations was set at pH 7, according to their pK_a values in water; the TPQ cofactor was assumed to be negatively charged, according to its pK_a value of approx. 3 [31]. The electrostatic energy along the channel of ECAO and PSAO was calculated using a finite-difference Poisson–Boltzmann method implemented in the Program UHBD [32]. An ionic strength of 100 mM and dielectric constants of 2 and 78 for the protein and the solvent environment respectively were used.

RESULTS AND DISCUSSION

Dependence of kinetic parameters on substrate structure

The chemical structure, the pK_a values of the amino groups [33,34] and the amine chain length of the studied substrates are reported in Table 1, in which the C and N atoms in the main chain are numbered according to their position with respect to

Table 1 Structure, chain length and pK_a values of amines used as BSAO substrates

The position number and the chain length (Å) are relative to the reacting amino group. The known pK_a values [33,34] are reported below the corresponding amino group (in the case of N^8-acetylspermidine, the pK_a values of spermidine were assumed). Any change in length due to the substitution of an N atom for a C atom in the linear chain (less than 5%) was not considered.

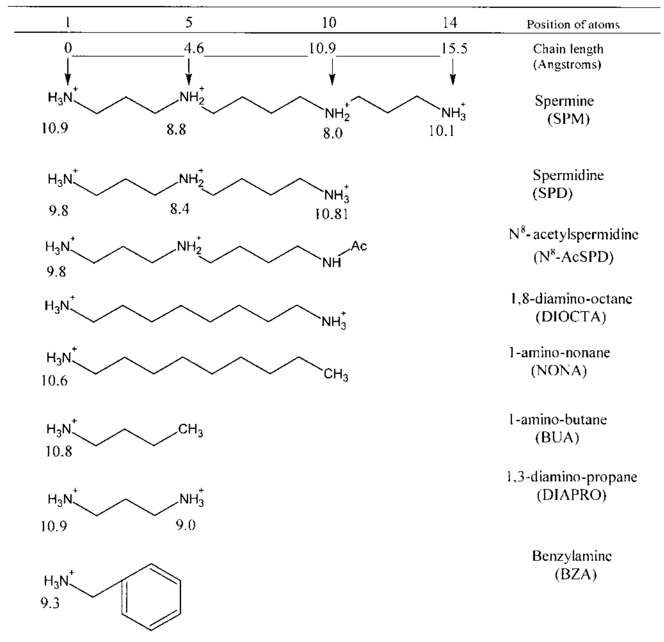


Table 2 Kinetic parameters of BSAO for various substrates at pH 7.20

The kinetic measurements were carried out in 25 mM Hepes containing 150 mM NaCl, at 37 °C, under saturation conditions for oxygen, using the amines reported in Table 1. All measurements were run in triplicate, and S.D. values for k_{cat} and K_m were ⩽ 15% in all cases.

Substrate	k_{cat} (s^{-1})	K_m (μM)	$10^{-3} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
Spermine	2.1	20	105
Spermidine	1.8	225	8.00
N^8-Acetylspermidine	0.6	640	0.94
1,8-Diamino-octane	0.9	0.36	2500
1-Aminononane	0.4	8.7	46.0
1-Aminobutane	1.2	2500	0.48
1,3-Diaminopropane	0.3	17 000	0.02
Benzylamine	0.9	1500	0.60

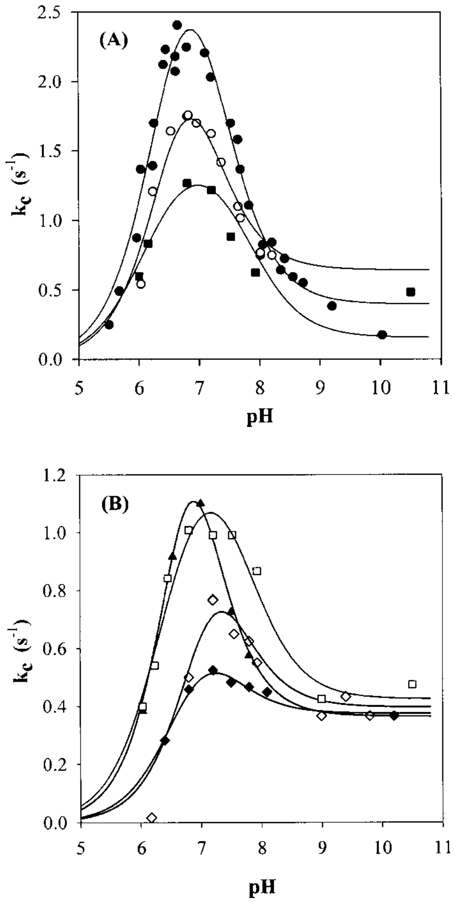
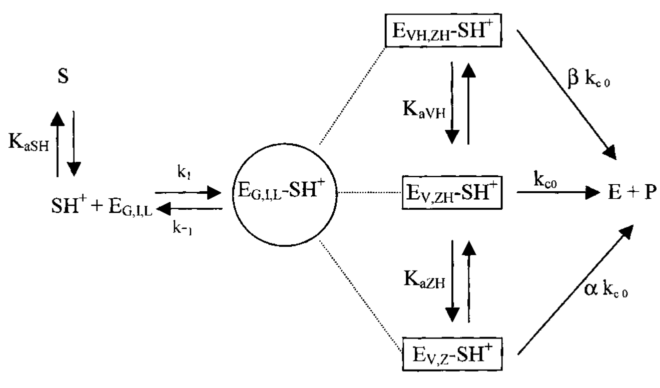


Figure 1 pH profiles of k_{cat} (k_c) values for some substrates of BSAO

Substrates investigated were: (**A**) spermine (●), spermidine (○), 1-aminobutane (■); (**B**) 1-aminononane (◆), 1,8-diamino-octane (▲), benzylamine (□) and N^8-acetylspermidine (◇). The solid lines were obtained by a non-linear least-squares fit of the experimental data to eqn (3). All experiments were carried out at constant ionic strength (162 mM) and at 37 °C, under saturation conditions for oxygen. For other experimental conditions, see the Experimental section.



Scheme 1 Kinetic scheme for the action of BSAO

See the text for details.

the reactive amino group. The kinetic parameters for the various substrates, calculated from experiments carried out at pH 7.2, are reported in Table 2. The following points are evident from

Table 3 Values of parameters controlling the chemical step of oxidative deamination catalysed by BSAO

The pK_a values of the residues controlling the chemical step for the various substrates, together with $k_{cat,o}$ and α values, were obtained by fitting the experimental data reported in Figure 1 to eqn (3). $\beta < 0.05$ was obtained by the fittings.

Substrate	p$K_{a,VH}$	p$K_{a,ZH}$	$k_{cat,o}$ (s^{-1})	α
Spermine	6.3 ± 0.1	7.6 ± 0.1	3.36 ± 0.32	0.0
Spermidine	6.4 ± 0.2	7.5 ± 0.2	2.66 ± 0.41	0.0
N^8-Acetylspermidine	6.8 ± 0.4	7.4 ± 0.3	1.32 ± 0.18	0.3
1,8-Diamino-octane	6.6 ± 0.2	7.3 ± 0.3	2.11 ± 0.57	0.0
1-Aminononane	6.6 ± 0.1	7.3 ± 0.3	0.75 ± 0.03	0.5
1-Aminobutane	6.3 ± 0.4	7.7 ± 0.2	1.58 ± 0.21	0.1
Benzylamine	6.4 ± 0.2	7.7 ± 0.4	1.41 ± 0.14	0.3

Table 2. (1) The k_{cat} values span less than one order of magnitude (from 0.3 to 2.1 s^{-1}), indicating that changes in the substrate structure remote from the carbon centre undergoing oxidation do not affect substantially the catalytic constant, i.e. the chemical step controlling the reaction. (2) The K_m values vary by around five orders of magnitude according to substrate structure, with consequent changes in k_{cat}/K_m (e.g. an increase of more than five orders of magnitude from 1,3-diaminopropane to 1,8-diamino-octane). In particular it appears that: (a) the affinity of BSAO increases (K_m decreases) with the chain length of the substrate; (b) the presence of a positively charged amino group at position 5 of amines with similar chain length decreases their affinity for BSAO by up to three orders of magnitude; and (c) the presence in a substrate of a positively charged group at position 10 increases its affinity for BSAO; for example, 1,8-diamino-octane compared with 1-aminononane, or spermidine compared with N^8-acetylspermidine. This strong dependence of K_m and k_{cat}/K_m values on substrate structure remote from the carbon centre undergoing oxidation suggests that these kinetic parameters depend on physical interactions between the substrate and the enzyme active site.

Dependence of k_{cat} on pH

The k_{cat} values for the substrates listed in Table 1 were calculated for the pH range 5.6–10.2, and are reported in Figure 1. In this study, 1,3-diaminopropane was not considered further because of the low k_{cat} and k_{cat}/K_m values. All the plots of Figure 1 show a similar bell-shaped profile, characterized by a maximum at pH 7.1 ± 0.2.

The pK_a values of the protonated groups (that we named VH and ZH), which appear to control the dependence of k_{cat} on pH, were calculated by fitting the experimental data to the following equation:

$$k_{cat} = k_{cat,o}\frac{(1+\alpha K_{a,ZH}/[H^+]+\beta[H^+]/K_{a,VH})}{(1+[H^+]/K_{a,VH}+K_{a,ZH}/[H^+])} \quad (3)$$

where $k_{cat,o}$ is the pH-independent catalytic constant, and $K_{a,VH}$ and $K_{a,ZH}$ are the acid dissociation constants of VH and ZH respectively.

This equation, obtained by Tipton and Dixon for 'a simplified reaction scheme' [35], contains the β and α factors introduced by Koudelka et al. [36] to take into account the possibility of non-zero activity of the fully protonated and deprotonated enzyme–substrate intermediates at low and high pH values respectively (see also Scheme 1). The results of the best fits of the experimental data to eqn (3) are shown in Figure 1 (continuous lines) and Table 3. From Table 3 it appears that the average pK_a values of

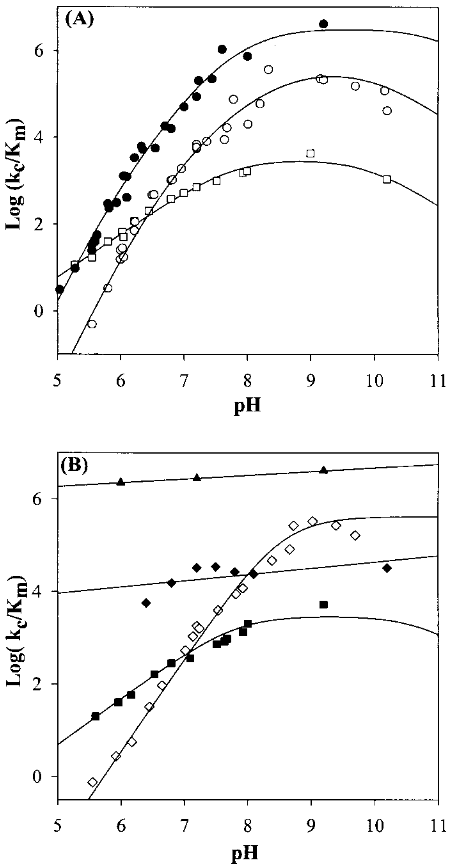


Figure 2 pH profiles of k_{cat}/K_m values for some substrates of BSAO

Shown are plots of log k_{cat} (k_c)/K_m against pH for: (**A**) spermine (●), spermidine (○), benzylamine (□); (**B**) N^8-acetylspermidine (◇), 1-aminobutane (■), 1-aminononane (◆) and 1,8-diamino-octane (▲). The solid lines were obtained by fitting the experimental data to eqn (5). For experimental conditions, see the legend to Figure 1.

the VH (6.5 ± 0.3) and ZH (7.50 ± 0.2) groups are independent of substrate structure, and are close to those reported for the BSAO/benzylamine system [5].

Dependence of k_{cat}/K_m on pH

Figure 2 indicates a very strong dependence of k_{cat}/K_m on pH and on substrate structure (differences of up to six orders of magnitude), except for 1-aminononane and 1,8-diamino-octane. Furthermore, at a given pH, a strong dependence of k_{cat}/K_m value on the structure of the substrate was observed (approx. seven orders of magnitude between spermidine and 1,8-diamino-octane at pH 5.5).

The average slopes [$\Delta(\log k_{cat}/K_m)/\Delta$pH] of the plots of the 'pH-sensitive substrates' (spermine, spermidine, N^8-acetylspermidine, 1-aminobutane and benzylamine) were calculated for the pH ranges 5–6 and 6.5–7.5, and are reported in Table 4. In the pH range 6.5–7.5, the slope of ≈ 2 found for spermine, spermidine and N^8-acetylspermidine, and the slope of ≈ 0.8 found for benzylamine and 1-aminobutane, strongly suggest that protonable groups control the interaction of these substrates with the active site of BSAO. In particular, in the case of spermine and spermidine, the value $\Delta(\log k_{cat}/K_m)/\Delta$pH of $\geqslant 3$ at pH < 6 strongly supports the hypothesis that k_{cat}/K_m values are controlled by three protonated residues (in this paper indicated as GH, IH and LH) of the active site. For the other substrates, the lower values of the maximum slope [$\Delta(\log k_{cat}/K_m)/\Delta$pH value of ≈ 2 for N^8-acetylspermidine and of ≈ 1 for 1-aminobutane and benzylamine] suggest that two groups (GH and IH) in the case of N^8-acetylspermidine, and one group (GH) in the case of 1-aminobutane and benzylamine, are involved in the control of k_{cat}/K_m.

Based on these data, a minimal reaction mechanism could be written for the 'pH-sensitive substrates', using the following reasonable assumptions: (i) the protonation of groups G, I and L occurs independently; (ii) the protonation and deprotonation steps are not rate limiting [37]; (iii) the reactive amino group of the substrate (N-1 atom) must be protonated to enter into the enzyme active site [5,17,20]; (iv) the binding of 1-aminobutane or benzylamine and of N^8-acetylspermidine occurs independently of the charge of the IH and LH groups and of the LH group respectively; and (v) the docking of the substrates occurs independently of the protonation state of the VH and ZH groups involved in the chemical step.

In particular for substrates with three (N-1, N-5 and N-10) or more protonated amino groups, such as spermine and spermidine, the mechanism shown in Scheme 1 can be envisaged. In Scheme 1, $K_{a,SH}$ is the acid dissociation constant of the reacting amino group of the substrate (the N-1 atom). $E_{G,I,L}$ is the unprotonated form of the enzyme, which is able to dock the protonated substrate (SH^+) to generate the corresponding enzyme–substrate complex, $E_{G,I,L}$–SH^+. Formation of the intermediate $E_{G,I,L}$–SH^+ is followed by the chemical reaction controlled by the VH and ZH groups. $E_{G,I,L}$–SH^+ (circular box), which shows the protonation states of GH, IH and LH, and $E_{VH,ZH}$–SH^+, $E_{V,ZH}$–SH^+ and $E_{V,Z}$–SH^+ (rectangular boxes), which show the protonation states of the VH and ZH groups, represent the same intermediate in the various protonation states. As above reported, α and β are the Koudelka factors.

Starting from Scheme 1 and introducing the steady-state approximation for the enzyme–substrate intermediates (see the Appendix for the kinetic treatment), we obtained the following rate law for spermine and spermidine:

$$V = \frac{k_{cat,o}\dfrac{(1+\alpha K_{a,ZH}/[H^+]+\beta[H^+]/K_{a,VH})[E_o][S]}{(1+[H^+]/K_{a,VH}+K_{a,ZH}/[H^+])}}{\dfrac{K_{m,o}A}{(1+[H^+]/K_{a,VH}+K_{a,ZH}/[H^+])}+[S]} \quad (4)$$

where $K_{m,o}$ is the pH-independent value of K_m, $K_{a,LH}$, $K_{a,IH}$ and $K_{a,GH}$ are the dissociation constants of the LH, IH and GH groups respectively, and A is defined as follows:

$$\begin{aligned} A = \{&1+([H^+]/K_{a,GH})+([H^+]/K_{a,IH})+([H^+]/K_{a,LH}) \\ &+([H^+]^2/K_{a,GH}K_{a,IH})+([H^+]^2/K_{a,GH}K_{a,LH}) \\ &+([H^+]^2/K_{a,LH}K_{a,IH})+([H^+]^3/K_{a,GH}K_{a,IH}K_{a,LH})\} \\ &\times[1+(K_{a,SH}/[H^+])] \end{aligned}$$

From the comparison of the Michealis–Menten equation with eqn (4), it appears that the apparent k_{cat}/K_m is given by:

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_o[1+\alpha(K_{a,ZH}/[H^+])+\beta([H^+]/K_{a,VH})]}{A} \quad (5)$$

where $(k_{cat}/K_m)_o$ represents the pH-independent value of catalytic efficiency.

The experimental data for the BSAO/spermidine and BSAO/spermine systems were fitted (continuous lines in Figure 2) to

Table 4 Slopes of plots of $\log(k_{cat}/K_m)$ against pH

The slopes for the 'pH-sensitive substrates' were calculated by linear regression ($r \geqslant 0.98$) in the pH ranges 5–6 and 6.5–7.5, from the data reported in Figure 2. Results are means ± S.D.

Substrates	Experimental slope $\{\Delta[\log(k_{cat}/K_m)]/\Delta pH\}$	
	pH 5–6	pH 6.5–7.5
Spermine, spermidine	3.1 ± 0.1	1.9 ± 0.2
N^8-Acetylspermidine	1.6 ± 0.3	1.9 ± 0.2
1-Aminobutane, benzylamine	1.2 ± 0.2	0.8 ± 0.2

Table 5 pK_a values of the GH and IH groups of BSAO involved in the docking of various substrates

The pK_a values were obtained by fitting the data of the plots of $\log(k_{cat}/K_m)$ against pH (Figure 2) to eqn (5).

Substrate	p$K_{a,GH}$	p$K_{a,IH}$
Benzylamine	8.0 ± 0.1	–
1-Aminobutane	8.1 ± 0.1	–
Spermidine	8.0 ± 0.4	7.7 ± 0.4
Spermine	8.3 ± 0.3	7.5 ± 0.3
N^8-Acetylspermidine	8.7 ± 0.5	8.3 ± 0.6

eqn (5), and the p$K_{a,GH}$ and p$K_{a,IH}$ values obtained are reported in Table 5 (the p$K_{a,SH}$ values shown in Table 1 were used for the fitting).

For the systems BSAO/N^8-acetylspermidine, BSAO/benzylamine and BSAO/1-aminobutane, a good fit (Figure 2) was obtained by fitting the experimental data to eqn (5), with A in the case of N^8-acetylspermidine defined as follows:

$$A = (1+[H^+]/K_{a,GH}+[H^+]/K_{a,IH}+[H^+]^2/K_{a,GH}K_{a,IH}) \times (1+K_{a,SH}/[H^+])$$

and, in the case of benzylamine and 1-aminobutane, as:

$$A = (1+[H^+]/K_{a,GH}) \times (1+K_{a,SH}/[H^+])$$

These equations were obtained by taking into account that only two residues (GH and IH) are involved in docking of N^8-acetylspermidine and only one residue (GH) is involved in docking of benzylamine and 1-aminobutane.

On the basis of the data reported in Table 5, we can conclude that two residues of the active site, characterized by p$K_{a,GH}$ = 8.2 ± 0.3 and p$K_{a,IH}$ = 7.8 ± 0.4, are involved in the control of substrate docking, in addition to the LH residue (p$K_{a,LH}$ = 6.2 ± 0.2) that is involved in the competitive binding of cations [38].

In the case of 1-aminononane and 1,8-diamino-octane (Figure 2), we found that $\Delta(\log k_{cat}/K_m)/\Delta pH$ gave a value of $\leqslant 0.2$ over the entire pH range explored. This lack of sensitivity of 1,8-diamino-octane and 1-aminononane to pH strongly suggests that the interaction of the long aliphatic chains of these substrates with a hydrophobic region of the active site controls their docking. This behaviour is in agreement with the presence of a hydrophobic region in the BSAO active site, as found for other amine oxidases [14,16,21,39].

Table 6 $2CZ_aZ_b$ values as a function of substrate structure

The $2CZ_aZ_b$ values were calculated, according to eqn (6), by linear regression of $\log(k_{cat}/K_m)$ for the various substrates against $I^{1/2}$, at various pH values (6.0, 7.2, 9.2 and 10.2). A correlation coefficient of > 0.98 was obtained for $|CZ_aZ_b| \geqslant 1$. Results are means ± S.D.

Substrates	CZ_aZ_b
Spermine*, spermidine	−3.7 ± 0.3
N^8-Acetylspermidine*, 1-aminobutane, benzylamine*	−1.3 ± 0.2
1-Aminononane, 1,8-diamino-octane	−0.2 ± 0.1

* In the case of these substrates, measurements were carried out only at pH 7.2.

Dependence of k_{cat} and K_m on ionic strength

The dependence of k_{cat} and k_{cat}/K_m on ionic strength (I) was studied in the range I = 10–200 mM at pH 7.2, and, in the case of spermidine, 1-aminononane, 1,8-diamino-octane and 1-aminobutane, also at pH 6.0, 9.2 and 10.2. The kinetic data were analysed according to the Debye Hückel equation [40]:

$$\log k = \log k_o + 2CZ_aZ_b(I)^{1/2} \qquad (6)$$

where k is k_{cat} or k_{cat}/K_m, Z_a and Z_b are the charges of the species involved in the formation of the enzyme–substrate complex, and k_o is the value of the kinetic rate constant at $I = 0$. The value of the constant C is 0.523 in water at 37 °C.

From analysis of the kinetic data obtained at various ionic strength values, we found that: (1) the k_{cat} values of the substrates tested were independent of ionic strength in the pH range 6–10 (results not shown); (2) the values of $\log(k_{cat}/K_m)$ are linearly dependent on $(I)^{1/2}$; and (3) no significant change in the $2CZ_aZ_b$ value with pH for a given substrate was observed (see Table 6).

According to the values of $2CZ_aZ_b$ reported in Table 6, the substrates can be grouped into three classes: class I, spermine and spermidine ($2CZ_aZ_b \approx -3.7$); class II, 1-aminobutane, benzylamine and N^8-acetylspermidine ($2CZ_aZ_b \approx -1.3$); class III, 1-aminononane and 1,8-diamino-octane ($2CZ_aZ_b \approx 0$). These data strongly suggest that electrostatic forces control the interactions between the substrates of classes I and II and BSAO. Conversely, no electrostatic interaction seems to be involved in the docking of 1-aminononane or 1,8-diamino-octane with BSAO.

Structural analysis of crystal structures

To date, the crystal structures of copper amine oxidases from *E. coli*, *Arthrobacter globiformis*, pea seedlings and *Harsenula polymorpha* [23] have been completed, and these show a high degree of structural similarity, despite the low sequence identity [41]. The following common features of the crystallized enzymes match with the docking of the various substrates in the case of the BSAO active site. (a) The lining of part of the internal surface of the active-site channel, along the longitudinal axis, with apolar residues, together with the presence of water molecules inside the channel itself, suggests a binding site for hydrophobic substrates (1-aminononane and 1,8-diamino-octane). (b) The presence along the longitudinal axis of the channel of a gradient of electrostatic potential energy due to the presence of negative charges. The energy decreases moving from the protein surface towards the bottom of the channel, where the TPQ cofactor lies (Figure 3). In these profiles, the presence of a shoulder could

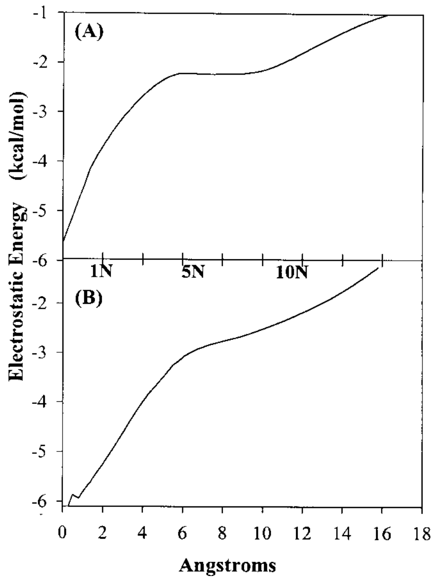


Figure 3 Electrostatic potential energy along the channel of ECAO (A) and PSAO (B)

The electrostatic energy (in kcal/mol; 1 kcal = 4.184 kJ) against channel length (in Å) is referred to an elementary positive charge used as a probe. 1N, 5N and 10N denote nitrogen atoms of a possible substrate.

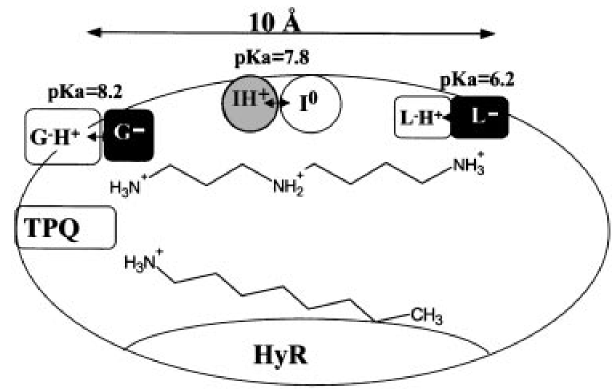


Figure 4 Active-site model of BSAO

This active-site model has been proposed on the basis of the experimental data. G^-H^+, IH^+ and L^-H^+ are residues that interact with the substrate according to its structure. The pK_a and charge of these groups and their approximate location are reported on the basis of experimental results. HyR is a hydrophobic region where highly hydrophobic substrates may bind. In this model, the two different binding modes are shown. Apolar substrates, such as nonylamine, interact with the hydrophobic binding region. On the other hand, polyamines (such as spermidine) interact, by electrostatic interactions, with the charged region of the active site where the three protonable residues (deprotonated in the enzyme active form), that control the docking of these polyamines, are localized.

be correlated with the presence of positively charged residues (Lys-296 in the case of PSAO and His-440 in the case of ECAO). This energy profile, if conserved in BSAO, could explain some of the features of the docking of the substrates in the BSAO active site.

Proposed model of the interactions controlling the docking of various substrates in the BSAO active site

Based on the dependence of BSAO activity on pH and ionic strength, and on the structural analysis of the Protein Databank structures, we have produced a schematic model of the BSAO active site (Figure 4). In this model, the presence of two different binding site regions for substrates is hypothesized. (1) The first is the binding site region for class I and II substrates, the docking of which is controlled mainly by electrostatic interactions. (2) The second binding site is the region where the docking of 1-aminononane and 1,8-diamino-octane (class III) should occur, controlled by hydrophobic forces. The freedom of the ring of TPQ to rotate within the active site [42–44] to achieve the correct orientation to interact with differently positioned and orientated substrates makes reasonable the hypothesis of two binding site regions.

Experimental data supporting the model in Figure 4 are as follows. (i) The substantial pH-independence of $2CZ_aZ_b$ values for a given substrate in the pH range 6–10, where, according to the dependence of k_{cat}/K_m values on pH, the pK_a values of groups G^-H^+, IH^+ and L^-H^+ lie. This behaviour indicates that the docking of spermine, spermidine, N^8-acetylspermidine, benzylamine and 1-aminobutane is favoured by electrostatic interactions between their positively charged N-1 atom and the G^- group and, in the case of spermidine and spermine (class I), also between the N-10 atom and the L^- residue. (ii) The presence in the active site of a negatively charged area due to the LH residue (pK_a 6.2), where cations bind competitively with spermine and spermidine [38]. (iii) The adverse effect on k_{cat}/K_m of a positively charged amino group at position 5 of the substrate indicates the presence in the active site of a group (IH^+) that may assume, according to the pH, a charge of $0/+1$. This group should play an 'on (charge = 0)/off (charge = +1)' role in the docking of substrates such as N^8-acetylspermidine, spermidine and spermine.

It should be underlined that residues GH, IH and LH have different roles in docking, depending on substrate structure and charge. The G^- group, responsible for the ionic interaction with the protonated N-1 atom of the substrate, could be the aspartate residue of the well conserved consensus sequence (Asp-300 in PSAO) [31]. Its high pK_a value (p$K_{a,GH} = 8.2 \pm 0.3$) is likely to be due to its localization in a hydrophobic region near the TPQ cofactor [31]. As this residue was also shown to be the catalytic base involved in proton abstraction from C-1 in the oxidative half-reaction [25], we cannot exclude the possibility that the VH group (p$K_{a,VH} = 6.5 \pm 0.3$), responsible for the pH-dependence of the k_{cat} values, and the GH group may be the same residue: the difference between the pK_a values might be explained by a different polarity of the environment in the presence and in the absence of the TPQ–substrate adduct.

The IH^+ group (pK_a 7.8 ± 0.4) could be tentatively identified as being an imidazole residue. In view of the distance between the N-1 and N-5 atoms of polyamines, this group should be localized approx. 6–8 Å from the reactive group of the TPQ cofactor, and may be responsible for an energy shoulder in the gradient of electrostatic energy (see Figure 3) that would also be present within the BSAO channel. As regards the L^-H^+ binding site, it may contain one or more carboxylic groups, as suggested previously [38].

Conclusions

In conclusion, the data presented herein show that substrates of BSAO may bind to different regions of the enzyme active site, depending on their structure and charge distribution. Furthermore, we propose that the docking of physiological polyamines (spermine and spermidine) is controlled by electrostatic interactions involving the two active-site residues facing the N-5 and N-10 atoms of the substrate, in addition to that described previously for N-1 [5]. These electrostatic interactions are likely to play a key role in the formation of the enzyme–substrate complex, since, under physiological conditions, the spermine concentration in plasma is approx. 0.03 μM [45], which is well below its K_m value.

These findings should provide improved insight into the structure–function relationships of BSAO and its substrates. These substrates are involved in numerous cellular functions, such as the control of protein and nucleic acid synthesis, the control of cell proliferation, differentiation and development [46,47], and involvement in detoxification and cell signalling processes. Knowledge of the factors controlling enzyme–substrate interactions should provide a basis for the design of specific regulators/inhibitors of mammalian amine oxidase, an area of intensive research.

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APPENDIX

Kinetic treatment of Scheme 1 for the BSAO/spermine and BSAO/spermidine systems

According to Scheme 1, the rate law for the formation of the reaction product (i.e. RCHO) from the reaction intermediates is:

$$v = dP/dt = k_{cat,o}[E_{V,ZH}\text{–}SH^+] + \alpha k_{cat,o}[E_{V,Z}\text{–}SH^+] + \beta k_{cat,o}[E_{VH,ZH}\text{–}SH^+] \quad (A1)$$

where SH^+ is the protonated substrate. From this equation, eqn (4) in the main text has been obtained by considering the following equations.

(i) The steady-state condition of the reaction intermediate ($E_{V,ZH}$–SH^+) evolving to the end products:

$$-d[E_{V,ZH}\text{–}SH^+]/dt = k_1[E_{G,I,L}][SH^+] - k_{-1}[E_{V,ZH}\text{–}SH^+] - k_{cat,o}[E_{V,ZH}\text{–}SH^+] \approx 0 \quad (A2)$$

(ii) The mass balance of the enzyme:

$$[E]_o = [E_{V,Z}\text{–}SH^+] + [E_{V,ZH}\text{–}SH^+] + [E_{VH,ZH}\text{–}SH^+] + [E_{G,I,L}] + [E_{GH,IH,LH}] + [E_{G,IH,LH}] + [E_{GH,I,LH}] + [E_{GH,IH,L}] + [E_{G,I,LH}] + [E_{GH,I,L}] + [E_{G,IH,L}] \quad (A3)$$

(iii) The mass balance of the substrate:

$$[S]_o = [SH^+] + [S] \quad (A4)$$

The terms $[E_{V,Z}$–$SH^+]$, $[E_{V,ZH}$–$SH^+]$ and $[E_{VH,ZH}$–$SH^+]$ can be ignored, since $[S]_o \gg [E]$.

(iv) The equations describing the acid–base equilibria of the substrate, of the GH, IH and LH groups of the free enzyme, and of the VH and ZH groups of the enzyme involved in the chemical mechanism:

$$K_{a,SH} = [H^+][S]/[SH^+] \quad (A5)$$

$$K_{a,GH} = [E_{G,IH,LH}][H^+]/[E_{GH,IH,LH}] \quad (A6)$$

$$K_{a,GH} = [E_{G,I,LH}][H^+]/[E_{GH,I,LH}] \quad (A7)$$

$$K_{a,GH} = [E_{G,IH,L}][H^+]/[E_{GH,IH,L}] \quad (A8)$$

$$K_{a,GH} = [E_{G,I,L}][H^+]/[E_{GH,I,L}] \quad (A9)$$

$$K_{a,IH} = [E_{GH,I,LH}][H^+]/[E_{GH,IH,LH}] \quad (A10)$$

$$K_{a,IH} = [E_{G,I,LH}][H^+]/[E_{G,IH,LH}] \quad (A11)$$

$$K_{a,IH} = [E_{G,I,L}][H^+]/[E_{G,IH,L}] \quad (A12)$$

$$K_{a,IH} = [E_{GH,I,L}][H^+]/[E_{GH,IH,L}] \quad (A13)$$

$$K_{a,LH} = [E_{GH,IH,L}][H^+]/[E_{GH,IH,LH}] \quad (A14)$$

$$K_{a,LH} = [E_{GH,I,L}][H^+]/[E_{GH,I,LH}] \quad (A15)$$

$$K_{a,LH} = [E_{G,IH,L}][H^+]/[E_{G,IH,LH}] \quad (A16)$$

$$K_{a,LH} = [E_{G,I,L}][H^+]/[E_{G,I,LH}] \quad (A17)$$

$$K_{a,VH} = [E_{V,ZH}\text{–}SH^+][H^+]/[E_{VH,ZH}\text{–}SH^+] \quad (A18)$$

$$K_{a,ZH} = [E_{V,Z}\text{–}SH^+][H^+]/[E_{V,ZH}\text{–}SH^+] \quad (A19)$$

Starting from these equations, a brief guide to the steps used to obtain the rate law (eqn 4 of the main text) is given. From eqn (A2), a new equation (eqn A2′) was obtained by substituting the concentration $[SH^+]$, expressed in terms of $[S]_o$ using eqns (A4) and (A5). From eqn (A3), a new equation (eqn A3′) was obtained (i) by substituting the concentrations $[E_{V,Z}$–$SH^+]$ and $[E_{VH,ZH}$–$SH^+]$ expressed in terms of the unknown $[E_{V,ZH}$–$SH^+]$ using eqns (A18) and (A19); and (ii) by substituting the concentrations of the various protonated forms of the free enzyme ($[E_{GH,IH,LH}]$, $[E_{G,IH,LH}]$, $[E_{GH,I,LH}]$, $[E_{GH,IH,L}]$, $[E_{G,I,LH}]$, $[E_{GH,I,L}]$ and $[E_{G,IH,L}]$) expressed in terms of the unknown $[E_{G,I,L}]$ using eqns (A6)–(A9), (A12), (A16) and (A17).

By this procedure, a system composed of two new equations (A2′ and A3′) was obtained:

$$k_1[E_{G,I,L}][S]_o/(1 + K_{a,SH}/[H^+]) - k_{-1}[E_{V,ZH}\text{–}SH^+] - k_{cat,o}[E_{V,ZH}\text{–}SH^+] = 0 \quad (A2')$$

$$[E]_o = \{1 + (K_{a,ZH}/[H^+]) + ([H^+]/K_{a,VH})\}[E_{V,ZH}\text{–}SH^+] + [E_{G,I,L}] \times \{1 + ([H^+]/K_{a,LH}) + ([H^+]/K_{a,GH}) + ([H^+]/K_{a,IH}) + ([H^+]^2/K_{a,LH}K_{a,GH}) + ([H^+]^2/K_{a,IH}K_{a,GH}) + ([H^+]^2/K_{a,LH}K_{a,IH}) + ([H^+]^3/K_{a,LH}K_{a,GH}K_{a,IH})\} \quad (A3')$$

This system contains the unknowns $[E_{V,ZH}$–$SH^+]$ and $[E_{G,I,L}]$, the known values $[H^+]$, $[E]_o$, $[S]_o$, $K_{a,SH}$, $K_{a,VH}$, $K_{a,ZH}$ and $K_{a,LH}$ (the constants $K_{a,VH}$, and $K_{a,ZH}$ were obtained from the k_{cat} measurements, and the constant $K_{a,LH}$ from [38]). Furthermore, in the above-reported equation system, the parameters $K_{a,GH}$, $K_{a,IH}$ and $k_{cat,o}/K_{m,o}$ [where $K_{m,o} = (k_{-1} + k_{cat,o})/k_1$] are present, the values of which have been calculated fitting the experimental data to eqn (5) of the main text. The system was solved by obtaining an expression of $[E_{V,ZH}$–$SH^+]$ in terms of the knowns and of the above-stated parameters $K_{a,GH}$, $K_{a,IH}$ and $k_{cat,o}/K_{m,o}$. The concentrations of the enzyme intermediates, obtained by using the expression $[E_{V,ZH}$–$SH^+]$ and eqns (A18) and (A19), were substituted into eqn (A1), obtaining eqn (4) of the main text.

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