Binding of Cations of Group IA and IIA to Bovine Serum Amine Oxidase: Effect on the Activity

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ABSTRACT   In this paper, we report on the presence of cation binding areas on bovine serum amine oxidase, where metal ions of the groups IA and IIA, such as Na^+, K^+, Cs^+, Mg^{2+}, and Ca^{2+}, bind with various affinities. We found a cation-binding area that influences the enzyme activity if occupied, so that the catalytic reaction may be altered by some physiologically relevant cations, such as Ca^{2+} and K^+. This binding area appears to be localized inside the enzyme active site, because some of these cations act as competitive inhibitors when highly charged amines, such as spermine and spermidine, are used as substrates. In particular, dissociation constant values (K_d) of 23 and 27 mM were measured for Cs^+ and Ca^{2+}, respectively, using, as substrate, spermine, a polyamine of plasma. An additional cation-binding area, where metal ions such as Cs^+ ($K_d \cong 0.1$ mM) and Na^+ ($K_d \cong 54$ mM) bind without affecting the enzyme activity, was found by NMR.

INTRODUCTION

Copper-containing amine oxidases (amine oxygen oxidoreductase deaminating, copper containing; EC 1.4.3.6) are a family of enzymes found in a variety of organisms (mammals, plants, bacteria, and yeasts) (Knowles and Dooley, 1994; Klinman and Mu, 1994; Tipping and McPherson, 1995; Medda et al., 1995).

Recently, the crystal structures of amine oxidases from *Escherichia coli*, pea seedling, *Harsenula polymorpha*, and *Arthrobacter globiiformis* have been solved (McGuirl and Dooley, 1999). The analysis of these structures shows considerable homology between the prokaryotic and eukaryotic enzymes. In particular, in addition to the well conserved copper site, the presence of a second type of metal-binding area has been found (Parsons et al., 1995; Kumar et al., 1996). This binding area is outside the active site and is located at ~32–33 Å from the copper atom. This feature should be present in all the copper amine oxidases because two of the six ligands (two aspartic residues) involved in the metal binding are fully conserved in all the sequenced amine oxidases (Kumar et al., 1996). According to the crystal structure and to electron spin resonance and atomic absorption data, both Mn^{2+} and Ca^{2+} ions could be good candidates to bind to this second metal-binding area of amino oxidases (Wilce at al., 1997; Sebela et al., 1997, De Vries et al., 2000), although according to mass spectral analysis (Murray et al., 1999) only Ca^{2+} may bind to this area in amine oxidase from *E. coli*.

A structural role has been hypothesized for this area even if its function is still unknown (Parsons et al., 1995; Kumar et al., 1996). A third type of metal-binding site has been

identified by Plastino et al. (1999) into the *H. polymorpha* active site. According to UV-Vis and resonance Raman spectroscopic analysis they found that cations such as cesium ion, dimethylammonium ion, and ammonium ion bind in the proximity of the TPQ cofactor and of the active-site base (Asp 319 in *H. polymorpha*) (Plastino et al., 1999).

The effect of the cations on amine oxidase activity is not clear. In fact, Bardsley et al. (1973) reported on a competitive effect of K^+ on pig kidney amine oxidase at concentrations higher than 0.3 M, and recently, Padiglia et al. (2001) reported on a reversible noncompetitive inhibition ($K_i = 200$ mM) of pig kidney amine oxidase (PKAO) by sodium and lithium ions. They observed that the inhibition becomes irreversible if PKAO is frozen in the presence of these ions. Based on the data reported by Plastino et al. (1999), Padiglia et al. (2001) proposed that these metal ions probably coordinated with the carboxyl group of the Asp 300 (in PKAO), change the configuration of the TPQ cofactor to a nonactive form.

The aim of this paper is to investigate the effect of cations of groups IA and IIA on the activity of bovine serum amine oxidase (BSAO). Our results indicate that among the cations of these groups, Cs^+, Ca^{2+}, and Mg^{2+} are relatively strong competitive inhibitors of BSAO. This effect has been observed when physiological amines such as spermine and spermidine are used. Furthermore, using NMR spectroscopy, we have demonstrated the presence of additional cation-binding areas where Cs^+ and Na^+ bind strongly without affecting the enzyme activity.

MATERIALS AND METHODS

All chemicals were of the highest available quality and were used without further purification. The substrates used in this work were from Sigma-Aldrich (Milan, Italy).

Purification and specific activity measurements of bovine serum amine oxidase were carried out according to Vianello et al. (1992). The specific activity of the purified enzyme was 0.36 U/mg. The concentration of the

purified enzymes was determined according to the method of Bradford (1976).

Activity measurements

Initial rate measurements were performed according to a peroxidase-coupled assay (Di Paolo et al., 1994). A Perkin-Elmer Lambda-17 spectrophotometer was used. Unless otherwise stated, all the measurements were carried out at 25°C, in 25 mM Hepes, 150 mM NaCl, pH 7.20.

The following buffers were used in the appropriate pH range: 2-[*N*-morpholino]ethanesulfonic acid (Mes) (pH 5.6–6.6), 3-[*N*-morpholino]propanesulfonic acid (Mops) (pH 6.6–7.2), Hepes (pH 7.2–7.8), *N*-[2-hydroxyethyl]piperazine-*N*′-propanesulfonic acid (Hepps) (pH 7.8–8.6). The buffer concentration was 25 mM, and activity measurements, performed at the overlapping pH values, showed no significant influence of the type of buffer on k_c and K_m values. When necessary, constant ionic strength values were obtained by adding a suitable concentration of the chloride salts of groups IA and IIA. Under pseudo-first-order conditions, the concentration of substrate used was $[S] \leq K_m \times 10^{-1}$.

To eliminate the effect of small fluctuations of enzyme activity, a standard assay was carried out together with each set of rate measurements. The standard activity assay was performed using a solution containing 0.5 mM spermine, 25 mM Hepes, 150 mM NaCl, pH 7.20. The ratio of the observed rate to 0.36 U/mg provided a correction factor applied to all the measurements within a given set of experiments.

Solubility of CaF$_2$ in the presence of polyamines

The solubility of CaF$_2$ ($pK_s = 10.40$, with K_s being the solubility product constant (Butler, 1964)) was measured stirring the solid salts for 24 h at 25°C in 20 mM Hepes, pH 7.2, in the presence and in the absence of 30 mM polyamine (spermine or 1,8-diaminooctane (DIOCTA)) and constant ionic strength (250 mM) by suitable addition of NaCl. The total concentration of Ca^{2+} in the equilibrated solutions was measured using the method of murexide (Williams and Moser, 1953). Citrate ($K_{dCa} \approx 3$ mM) was substituted for the polyamines to verify the effect of complex formation on the solubility of CaF$_2$.

NMR measurements

Relaxation times were measured in flat-bottom tubes (outer diameter, 5 mm) containing 250–280 μl of solution. The NMR measurements were carried out in 10 mM Hepes containing 10 mM NaCl and 10% D$_2$O, for field frequency lock, pH 7.20. The measurements were run at constant temperature (25°C). Samples were not spun, and shimming was performed on ^{1}H free induction decay (FID). The spectral widths were 2000 Hz for ^{133}Cs$^+$ and 1000 Hz for ^{23}Na$^+$ ion, respectively.

Fully relaxed ^{133}Cs spectra were acquired at 25°C, using a 60° pulse and 50-s relaxation delay. A solution of 20 mM CsBr in an external coaxial capillary was used as a chemical shift reference. A Bruker MSL 300 instrument operating at 39.4 and 79.4 MHz for ^{133}Cs and ^{23}Na, respectively, was used for these studies.

^{23}Na transversal relaxation times (T_2) measurements were performed by the Carr-Purcell-Gill-Meiboom (CPGM) pulse sequence. This sequence was used also to obtain the T_2 values of Cs$^+$ solutions at [Cs$^+$] 1 mM. At lower concentrations, the T_2 values were calculated from the half-height linewidth of the Cs$^+$ spectrum. To verify the agreement between the two procedures, parallel experiments were performed at 1 mM Cs$^+$, and very similar $1/T_2$ values were obtained. Relaxation delays of 1 s and 100 s were used for the T_2 measurements of ^{23}Na$^+$ and ^{133}Cs$^+$, respectively.

Circular dichroism measurements

Circular dichroism (CD) spectra were acquired with a Jasco J-710 spectropolarimeter (Jasco, Tokyo, Japan) at 25°C. The spectra in the UV range (195–260 nm) were carried out in 0.25 mM Hepes, pH 7.2, containing 0.11 mg/ml BSAO, using a 0.1-cm quartz cuvette and the following experimental conditions: bandwidth = 2 nm, time constant = 8 s, and scan rate = 20 nm min^{-1}. The CD spectra in the visible (Vis) range (310–540 nm) were carried out in a solution containing 10 mM Hepes, 10 mM NaCl, pH 7.2, and 4.8 mg/ml BSAO. A 1-cm quartz cuvette and the following instrumental setup were used: bandwidth = 2 nm, time constant = 2 s, and scan rate = 50 nm min^{-1}. Four scans were averaged and then corrected for background (buffer plus salt). Na$^+$, Cs$^+$, or Ca^{2+} were added as chloride salts.

Data analysis

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

RESULTS AND DISCUSSION

Rate of oxidative deamination of polyamines in the presence of cations of groups IA and IIA

The effect of cations of groups IA (Li$^+$, Na$^+$, K$^+$, Rb$^+$, and Cs$^+$) and IIA (Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}) on BSAO activity was tested using *N*-(3-aminopropyl)-1,4-butanediamine (spermidine, SPD), *N,N*′-bis(3-aminopropyl)-1,4-butanediamine (spermine, SPM), *N*-8-acetylspermidine (N8AcSPD), DIOCTA, 1-aminononane (nonylamine), 1,4-diaminobutane (putrescine), and benzylamine as substrates (S). Because the BSAO activity is sensitive to ionic strength (Stevanato et al., 1994), the experiments were performed at pH 7.20, in 25 mM Hepes, in the presence of 150 mM and 50 mM of chloride salts of cations of groups IA and IIA, respectively ($I = 0.15$ M in both cases). For some of the tested substrates, a clear dependence of the enzyme activity on the cation present in solution was found at substrate concentrations lower than K_m (pseudo-first-order conditions; Table 1). For all the amines tested, the maximum activity was obtained in the presence of Na$^+$ ion, and this activity was taken as reference value (100%). Based on Table 1, it appears that the highest inhibition effect is obtained when the substrate bears an amino group in position 10 with respect to the reacting NH$_2$ (in this case all the C and N atoms in the main chain of these substrates were considered). In fact, in the presence of 50 mM Mg^{2+} or Ca^{2+} or 150 Mm Cs$^+$ or Rb$^+$, the enzyme activity is strongly decreased when SPM, SPD, N8AcSPD, or DIOCTA is used as substrate.

The possibility that these cations, in particular the divalent cations, apparently affect the BSAO activity by complexing the amines was excluded in the case of Ca^{2+} ion, chosen as representative of the group IIA cations. In fact the addition of 30 mM polyamine (SPM or DIOCTA) to a slightly soluble salt such as CaF$_2$ did not increase the total concentration of Ca^{2+} in solution over that predicted by the K_s alone. From these experimental data and taking into account the precision of the method we used to measure the Ca^{2+} concentration in solution (SD < 10%), it was calcu-

TABLE 1 Activity of BSAO in the presence of various cations

Substrate	$K_{m_{Na}}$ (μM)*	Relative k_c/K_m[†]	Relative amine oxidase activity[‡]					
			Li$^+$	K$^+$	Rb$^+$	Cs$^+$	Mg^{2+}	Ca^{2+}
SPM	10	100	75	72	40	16	51	53
SPD	200	14	75	72	35	20	73	55
N8AcSPD	330	0.7	§	§	§	27	40	49
BZA	1000	0.2	§	§	§	§	§	§
DIOCTA	0.5	1170	§	§	§	§	63	55
PUT	24,000	8×10^{-4}	§	§	§	48	§	§
NONA	8	25	§	§	§	§	§	§

The experiments were performed at pH 7.20, in 25 mM Hepes, in the presence of 150 mM and 50 mM chloride of cations of groups IA and IIA, respectively.
*The reported values were calculated for the various substrates in the presence of 150 mM NaCl, 25 mM Hepes, at pH 7.20.
[†]The k_c/K_m of spermine (2.4×10^7 M^{-1} min^{-1}) in the presence of 150 mM NaCl was taken as 100%.
[‡]The activity in the presence of 150 mM NaCl was taken as 100%.
§Residual activity higher than 80%.

lated that, if the Ca^{2+} forms a complex with SPM or DIOCTA, the dissociation constant for the complex must be higher than 300 mM. This value is at least an order of magnitude higher than the K_d value of the complex BSAO-cation calculated by activity measurements (next section).

As none of the tested cations affect BSAO activity under saturation conditions, that is at [S] $\gg K_m$, the preliminary results of Table 1 suggest that Li$^+$, K$^+$, Rb$^+$, Cs$^+$, Li$^+$, Ca^{2+}, and Mg^{2+} might behave as competitive inhibitors of BSAO. No specific effect of F$^-$, Cl$^-$, Br$^-$, I$^-$, or SO4^{2-}, on the k_c and K_m values of BSAO was observed in this study.

Cations of groups IA and IIA as competitive inhibitors of BSAO

Lineweaver-Burk plots were obtained from measurements of the initial rates of the oxidative deamination of SPM by BSAO in the presence of increasing concentrations of the ions reported in Table 1, that is Cs$^+$, Rb$^+$, K$^+$, Li$^+$, Mg^{2+}, and Ca^{2+}. In these experiments, the ionic strength was kept constant (215 mM) through the appropriate addition of NaCl, because in preliminary experiments, a $K_{dNa} \gg 1$ M was calculated by activity measurements. From these plots, it appears that all the considered ions behave as competitive inhibitors of BSAO, even if the effect of K$^+$, Li$^+$, and Rb$^+$ is small in comparison with the effect of Cs$^+$ and Ca^{2+}. As an example, see Fig. 1 *A*, where the Lineweaver-Burk plots obtained in the presence of increasing concentrations of Ca^{2+} are shown.

To calculate the dissociation constant of the complex of the enzyme with the cation M^{n+}, with M^{n+} being a cation of group IA or IIA, the Dixon's equation (Dixon, 1953; see Eq. 1) for a competitive inhibitor was used, assuming the cation to be a competitive inhibitor:

$$K_{m_M}/K_{m_{Na}} = (1 + [M^{n+}]/K_{d_M}), \qquad (1)$$

where K_{mM} and K_{mNa} are the apparent Michaelis constants obtained from measurements performed in solution contain-

ing Na$^+$ ions in the presence and in the absence of the cation M^{n+}, respectively, and K_{dM} is the dissociation constant of the complex enzyme-M^{n+}.

From the linear plots obtained according to Eq. 1 ($r > 0.996$; Fig. 1 *B)*, we calculated $K_{dM} = 278$ mM for K$^+$ and Li$^+$, $K_{dRb} = 156$ mM, $K_{dCs} = 23$ mM, $K_{dCa} = 27$ mM, and $K_{dMg} = 42$ mM. Because K_{dM} for Ba^{2+} and Sr^{2+} are relatively high, we calculated their values from the residual activity measured under the experimental conditions of Table 1 ($K_{dM} \sim 800$ and 600 M for Sr^{2+} and Ba^{2+}, respectively). In Fig. 2 the K_{dM} values are plotted as a function of the ionic radius of the tested cations (Cotton and Wilkinson, 1980). From this figure it appears that the K_{dM} values of the group IA cations are strongly affected by the ion dimensions, decreasing by an order of magnitude from K$^+$ to Cs$^+$. A similar dependence of affinity toward cations was found in a variety of biomolecules, such as nicotinic acetylcholine receptors (Akk and Auerbach, 1996), cubic insulin crystals (Badger et al., 1994), methylamine dehydrogenase (Kuusk and McIntire, 1994), and double-stranded DNA (Bleam et al., 1980). According to Eisenman (1961) the enhanced affinity increasing the ionic radius of cations occurs when the dehydration free energy of the ion is the dominant factor determining the free energy of transfer of the ion itself from the bulk to the local environment (from solution to BSAO active site in our case). In fact the dehydration free energy for monovalent cations decreases with the ionic radius.

In the case of group IIA cations, a clear cutoff size effect occurs (Fig. 2). In fact, it appears that the cavity size is such that it can accommodate only divalent cations with ionic radius lower than 1 Å, suggesting that the charge density of the ion should regulate the binding.

Furthermore, based on the K_{dM} value, we calculated $\Delta G^o_{Cs+} = -2.2$ kcal/mol and $\Delta G^o_{K+} = -0.77$ kcal/mol for the process of binding of Cs$^+$ and of K$^+$ to the BSAO active site. According to the Manning theory (Manning, 1984) ΔG^o values of the order of a few kilocalories per mole suggest a territorial association and short-range interactions
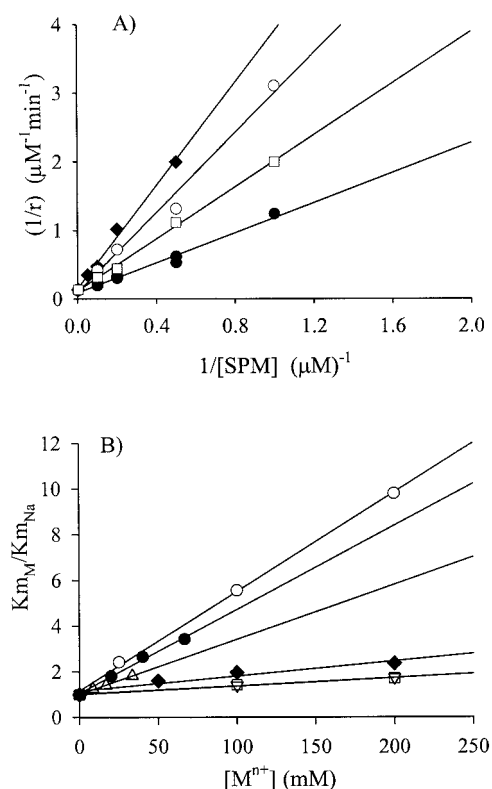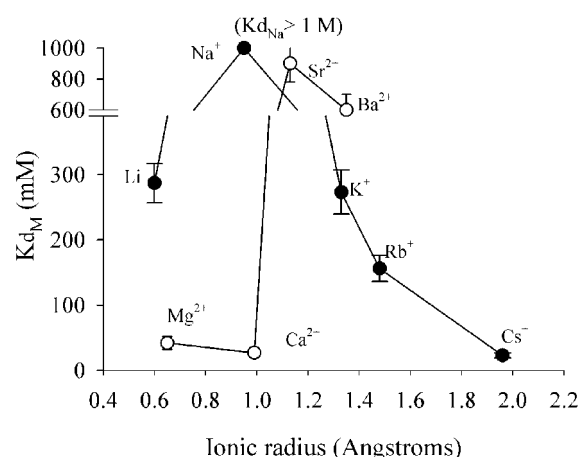
FIGURE 2 Dependence of the K_{dM} values of the complexes BSAO-metal ions on the ionic radius of cations. Most of the reported K_{dM} values were calculated from the data reported in Fig. 1 *B*.

FIGURE 1 Competitive inhibition of BSAO by cations. (*A*) Lineweaver-Burk plot showing the inhibition of BSAO by Ca^{2+}. The initial velocities (*r*) were measured as a function of spermine concentration at various concentrations of Ca^{2+} (in mM): ●, 0; □, 20; ○, 40; , ◆, 66.5. (*B*) Dependence of the ratio K_{mM}/K_{mNa} on the concentration of the cation M^{n+}: ●, Ca^{2+}; ○, Cs^{+}; □, K^{+}; △, Mg^{2+}; ◆, Rb^{+}; ▽, Li^{+}. The experiments were performed in 25 mM Hepes, pH 7.2. The ionic strength was adjusted to 215 mM by suitable addition of NaCl.

between the cation and the fully or partially negative surface of the binding site.

Ionic strength dependence of the BSAO activity in the presence of Na^{+}, Cs^{+}, and Ca^{2+} ions

The dependence of the spermine oxidase activity of BSAO on ionic strength (*I*) was studied using $CsCl$, $CaCl_2$, and $NaCl$ to vary the *I* values. These experiments were carried out at $[SPM] \ll K_m$. No experiment was carried out at $[SPM] \gg K_m$, as no effect of ionic strength on k_c was reported (Stevanato et al., 1994). Taking into account the inhibition effect of Cs^{+} and Ca^{2+} ion on BSAO activity, the Debye-Hückel equation (Atkins, 1986) was modified as follows:

$$\log(k_c/K_m) = \log(k_c/K_m)_o + 2CZ_aZ_bI^{1/2}$$
$$+ \log(1 + [M^{n+}]/K_{d_M}), \quad (2)$$

where Z_a and Z_b are the charges of the species involved in the formation of the enzyme-substrate complex, $(k_c/K_m)_o$ is

the catalytic efficiency at zero ionic strength, and *C* is a constant that depends on temperature, density, and dielectric constant of the medium (Leidler and Bunting, 1973). In water and at 25°C, *C* is 0.497 $M^{1/2}$. Fitting the experimental data to Eq. 2 and using the K_{dM} values obtained by the activity data (see previous section), we obtained the same value of $2CZ_aZ_b$ for the various cations, which is -4.3 ± 0.3 for Na^{+}, Cs^{+}, and Ca^{2+}. These results agree with our previous report (Stevanato et al., 1994). The independence of BSAO activity on the type of ion used to increase the ionic strength is in agreement with the Debye-Hückel theory.

pH dependence of BSAO activity on the presence of Na^{+}, Cs^{+}, Rb^{+}, Mg^{2+}, and Ca^{2+} ions

K_m and k_c values of BSAO at various pH values were calculated from Lineweaver-Burk plots using spermine or spermidine as substrate. The measurements were carried out in the pH range 5.6–7.6 in the presence of 150 mM Na^{+}, Rb^{+}, or Cs^{+} or of 50 mM Ca^{2+} or Mg^{2+}, which is at the same ionic strength (150 mM). The experiments were performed using both spermine as substrate in the presence of Na^{+}, Cs^{+}, or Ca^{2+} (Fig. 3 *A*) and spermidine as substrate in the presence of Na^{+}, Rb^{+}, or Mg^{2+} (Fig. 3 *B*). The results show that both k_c and K_m values depend on pH as found by Farnun et al. (1986) for the system BSAO-benzylamine. However, although the k_c values, both for SPM and SPD, at various pH values are independent of the type of cation (data not shown) the dependence of K_m values on pH is sensitive to the type of cation (see Fig. 3). Because Na^{+} does not interfere with the enzyme activity, to highlight the effect of pH on the metal binding, in this figure we reported the dependence of the ratio K_{mM}/K_{mNa} on pH, where M is Ca^{2+}, Cs^{+}, Rb^{+}, or Mg^{2+}. Because the ratio K_{mM}/K_{mNa}
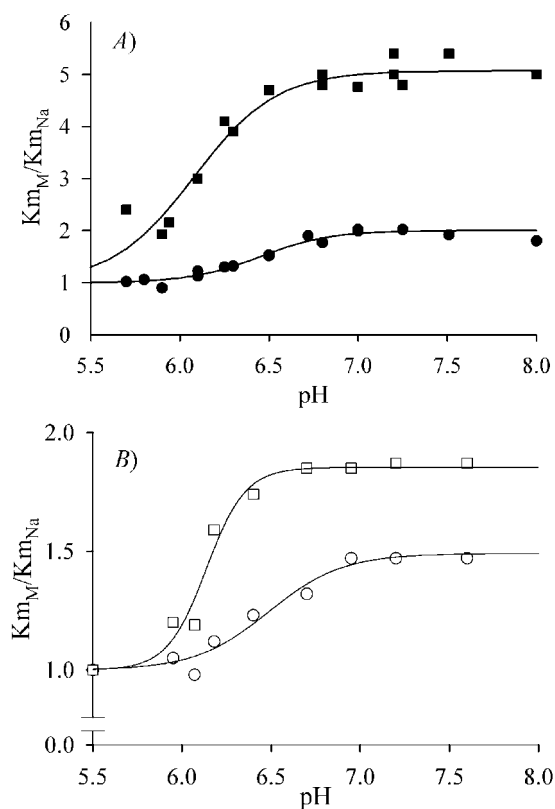
FIGURE 3 pH dependence of K_m of BSAO in the presence of Na$^+$, Ca^{2+}, Mg^{2+}, Rb$^+$, and Cs$^+$ ion. (A) Dependence of the ratios K_{mCs}/K_{mNa} (■) and of K_{mCa}/K_{mNa} (●) on pH, using spermine as substrate; (B) Dependence of the ratios K_{mMg}/K_{mNa} (□) and of K_{mRb}/K_{mNa} (○) on pH, using spermidine as substrate. The continuous lines were obtained by fitting the experimental results to Eq. 3.

behaves as a titration curve of an acid residue, the pK_a of the amino acid residues involved in the binding of the metal ions were calculated by fitting the experimental data from Fig. 3 to the Hill equation, modified by Markley (1973):

$$(K_{m_M}/K_{m_{Na}}) = a[1 + 10^{nHx(pKa-pH)}]^{-1}$$
$$+ c\{1 - [1 + 10^{nHx(pKa-pH)}]^{-1}\}, \quad (3)$$

where a and c are the values of the ratio (K_{mM}/K_{mNa}) measured when the metal binding site is deprotonated and protonated, respectively, and nH is the Hill coefficient, which is the number of the protons involved in the inhibition of cations. In Eq. 3, we assumed $c = 1$, because K_{mM} approaches close to K_{mNa}, decreasing the pH as can be seen in Fig. 3. From the fitting of the experimental results reported in Fig. 3 to Eq.3, similar values of the parameters pK_a and nH were calculated: $nH = 1.9 \pm 0.3$, and p$K_a = 6.2 \pm 0.2$. These results clearly indicate that the pK_a value of residues that are involved in the metal binding is invariant with the metal ions (monovalents and divalents) and the substrates. The value of $nH \approx 2$ suggests that two acid

residues are involved in the binding of these cations. Because the highest inhibitory effect of Cs$^+$, Rb$^+$, Mg^{2+}, and Ca^{2+} was observed in the presence of SPM and SPD (Table 1), these acid residues appear to be involved in the binding of the amino group present in position 10 of these polyamines. According to the distance between the 1N atom and 10N atom of polyamines, these acid residues should be localized at ~11–12 Å from the reactive group of TPQ cofactor, and as a consequence, they cannot be identified with the catalytic base of the amine oxidases (Asp319 in *H. polymorpha* (Plastino et al., 1999) or Asp300 in PKAO (Padiglia et al., 2001)).

NMR study of the interaction between BSAO and cesium and sodium ion

NMR measurements of chemical shift and of line broadening of ^{133}Cs and of ^{23}Na nuclei were carried out to obtain information on the binding of these cations to BSAO in the absence of the substrate. We decided to perform the experiments with these two monovalent ions because ^{23}Na$^+$ is the cation with the higher concentration present in plasma and ^{133}Cs$^+$ is considered a good NMR probe of intracellular environment (Shehan et al., 1995). Moreover, these two ions are characterized by high NMR sensitivity, large chemical shifts range, and narrow linewidths. Conversely, ions such as ^{43}Ca^{2+}, Mg^{2+}, and Mn^{2+}, which are very interesting from the biochemical point of view, are characterized by a low NMR sensitivity (Ca^{2+} and Mg^{2+}) or a high paramagnetism (^{55}Mn^{2+}). The NMR spectra of aqueous solutions of ^{23}Na$^+$ and ^{133}Cs$^+$, buffered at pH 7.2 and containing 0.1 mM BSAO, were single peaks in the range [Na$^+$]/[BSAO] = 100-5000 and [Cs$^+$]/[BSAO] = 2–200. The line broadening of these peaks increases, decreasing the ratio [cation]/[protein], as reported in Fig. 4, *A* and *B*. In Fig. 5 the spectra obtained at 1 mM Cs$^+$, in the presence and in the absence of 0.1 mM BSAO, are shown as examples. In these experiments, we observed negligible shifts of the resonance frequency of ^{23}Na$^+$ and ^{133}Cs$^+$ with respect to the frequency measured in the absence of BSAO.

The line shape of these peaks was fitted to a Lorentzian function, indicating a probable fast exchange of the cations between the two environments (bulk and protein binding site) (Sandström J., 1982).

Under the conditions of fast exchange, the observed transversal relaxation rate (R) is given by the following simplified equation (Bleam et al., 1980):

$$R = [(1/T_2)_{+BSAO} - (1/T_2)_{-BSAO}] = \chi_B R_B - \chi_F R_F, \quad (4)$$

where $(1/T_2)_{+BSAO}$ and $(1/T_2)_{-BSAO}$ are the experimental relaxation rates in the presence and in the absence of BSAO, χ_F and χ_B are the molar fraction, and R_B and R_F are the relaxation rate of the ion bound (B) to the protein and free ion (F), respectively. The line broadening of the Na$^+$ and
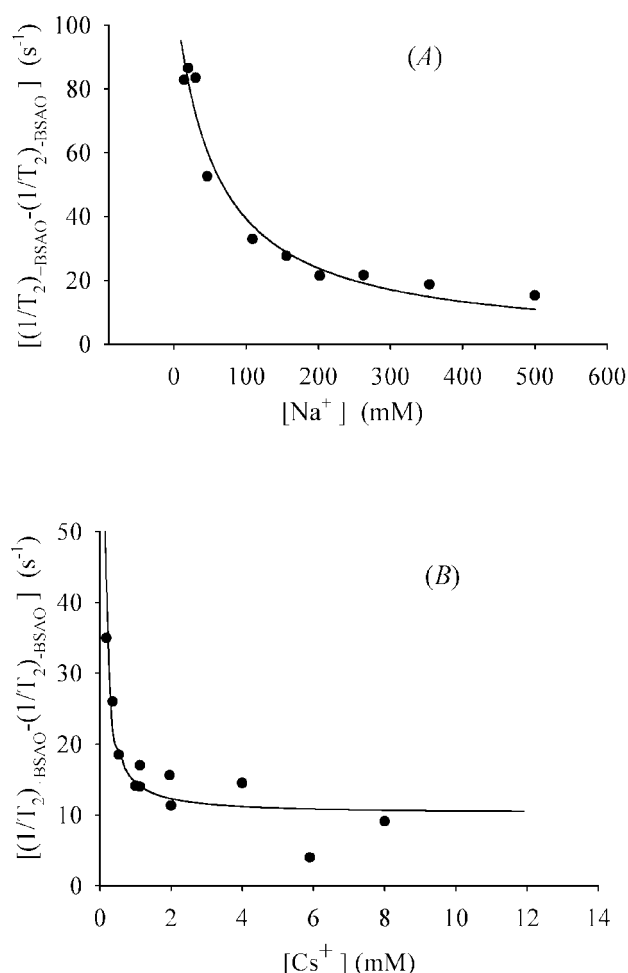
FIGURE 4   Effect of BSAO on transversal relaxation rates (*R*). (*A*) Dependence of $^{23}Na^+$ relaxation rate on Na^+ concentration; (*B*) Dependence of $^{133}Cs^+$ relaxation rate on Cs^+ concentration, in the presence of 10 mM NaCl. All of these experiments were carried out at pH 7.2 in the presence of 0.1 mM BSAO.

Cs^+ resonances by BSAO is not a trivial effect due to the protein presence, because we did not observe this behavior in the presence of bovine serum albumin. As representative examples, the *R* values of 8 mM $^{133}Cs^+$ were 12 s^{-1} in the presence of 18 mg ml^{-1} BSAO and ~0.2 s^{-1} in the presence of 18 mg ml^{-1} bovine serum albumin, respectively. Similarly, the *R* values of 14 mM $^{23}Na^+$ were 82.8 s^{-1} (with BSAO present) and 5.3 s^{-1} (with bovine serum albumin present).

The measurements of the relaxation rate were carried out at $[Na^+] \geq 10$ mM (see Fig. 4 *A*) to avoid precipitation of the enzyme. These experimental data were well fitted assuming the presence of only one type of Na^+-binding site on the enzyme. In particular, the transversal relaxation rates were fitted to the following equation (for more details, see Appendix):

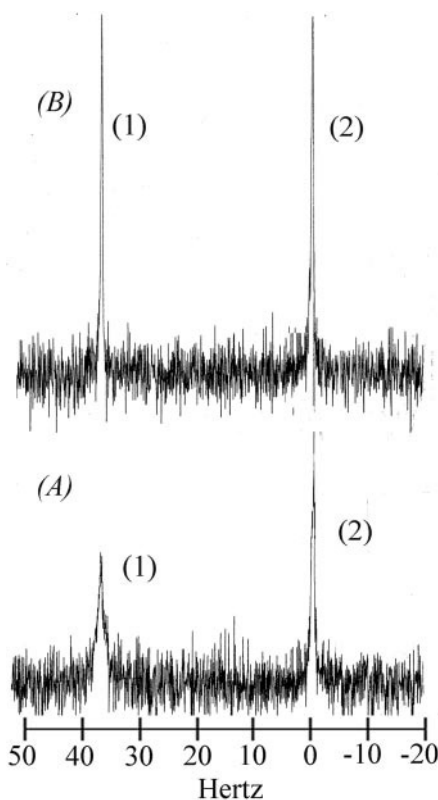$$R_{Na} = \{[E]_0/(K_{d_{Na}} + [Na^+]_0)\} \times R_{Na\text{-}E}, \qquad (5)$$

FIGURE 5   Line broadening of the NMR spectrum of $^{133}Cs^+$ ion induced by BSAO. (*A*) Spectrum of 1 mM Cs^+ (*peak 1*), in the presence of 0.1 mM BSAO; (*B*) Spectrum of 1 mM Cs^+, in the absence of the protein. The spectra were acquired in a solution buffered with 10 mM Hepes, pH 7.2. CsBr in an external coaxial capillary (*peak 2*) was used as a chemical shift reference.

where $[E]_0$ and $[Na^+]_0$ are the analytical concentrations of the enzyme and sodium ion, respectively, and $R_{Na\text{-}E}$ is the relaxation rate of the ion bound to the enzyme. The continuous line of Fig. 4 *A* is the fitting curve according to Eq. 5. Leaving K_d and $R_{Na\text{-}E}$ as floating parameters, we obtained the values reported in Table 2.

The relaxation measurements of the $^{133}Cs^+$ nucleus were performed in the presence of constant concentration of Na^+ ion (10 mM), varying the Cs^+ concentration in the range of 0.18–95 mM. A significant dependence of the line broadening on Cs^+ was found only at $Cs^+ < 4$ mM (Fig. 4 *B*). Furthermore, in a set of experiments performed at various Cs^+ concentrations and in the presence of 200 mM Na^+, we

TABLE 2   **Parameters characterizing the binding of Na^+ and Cs^+ ions to BSAO obtained by NMR measurements**

Nucleus	K_d (mM)	$R_{E\text{-}M}$ (s^{-1})
$^{23}Na^+$	53.6 ± 11.9	(6.67 ± 0.89) × 10^4
$^{133}Cs^+$	0.28 ± 0.11	10.9 ± 2.1

For $^{133}Cs^+$, 10 mM NaCl was added to the buffer to avoid the precipitation of BSAO; the other experimental conditions are reported under Materials and Methods.

found Cs^+ relaxation rates lower than those measured in the presence of 10 mM Na^+ (data not shown). On this basis we developed a scheme to model the interaction of Cs^+ with the enzyme. This scheme takes into account the competition between Cs^+ and Na^+ for the same binding site (see Appendix) and leads to the following equation:

$$R_{Cs} = \{A + [E]_0 + [Cs^+]_0 + \{(A + [E]_0 + [Cs^+]_0)^2$$
$$- 4[E]_0[Cs^+]_0\}^{1/2}\}\{(R_{E-Cs})/2[Cs^+]_0\}, \quad (6)$$

where $[Cs^+]_0$ is the analytical cesium concentration, R_{E-Cs} is the relaxation rate of Cs^+ bound to enzyme, and $A = K_{dCs}$ $(1 + [Na^+]_0/K_{dNa})$. In the second row of Table 2, we report the K_d and R_{E-Cs} values obtained by fitting the experimental data to Eq. 6 (continuous line of Fig. 4 *B*). The highest Cs^+ concentration used in the relaxation experiments was 95 mM, at both 10 and 200 mM Na^+. However, at $[Cs^+]$ 8 mM, the relaxation rate did not show any additional increase, because $\chi_F \approx 1$, and therefore the term $\chi_F R_F$ in Eq. 4 becomes dominant.

The comparison between the K_{dM} values of Na^+ and Cs^+ obtained by NMR (Table 2) and by activity measurements indicates that by NMR we are observing a different metal-binding site, probably located far from the enzyme active site.

Spectrophotometric studies of the interaction between BSAO and Cs^+, Ca^{2+}, and Na^+ ions

To investigate the effect of the cation binding on the secondary structure of BSAO, UV-CD spectra of native BSAO were carried out in the presence of 0.25–160 mM Na^+ and of 0.25–50 mM Cs^+ or Ca^{2+}. In these spectra, which are characteristic of α/β proteins, no significant change in molar ellipticity was observed.

To investigate the effect of a possible interaction of cations with the cofactor (TPQ) Vis-CD and absorption spectra of the native BSAO were performed, in the presence of 14–300 mM Na^+ and 1–100 mM Cs^+ or Ca^{2+}. This latter study was prompted by the results obtained with yeast amine oxidase, where cations such as cesium and dimethylammonium produce a shift of the absorbance maxima (in the range of 340–350 nm in the wild-type amine oxidase) because of the binding of these cations in the vicinity of the TPQ and active-site base (Plastino et al., 1999). In our case, no significant change in the molar ellipticity and no shift in the λ values of the major absorption bands of the Vis absorption spectrum was observed in the 310–600-nm range in experiments carried out at the same I values (data not shown). These results indicate that the binding of cations to BSAO, which we have demonstrated to occur by NMR and activity measurement, does not perturb significantly the secondary structure of the enzyme and the UV-Vis spectra characteristics of the TPQ cofactor.

In conclusion, the analysis of the activity and NMR data shows the presence on BSAO of two types of cation-binding sites, besides the Cu^{2+}-binding site.

According to the NMR investigation, Cs^+ and Na^+ bind to a site we call type 1, with a relatively high affinity (K_{dNa} = 54 mM and K_{dCs} = 0.28 mM), without affecting the enzyme activity. This site could be the external metal-binding site that appears on the recently published crystal structures of amine oxidases (Parsons et al., 1995; Kumar et al., 1996; Wilce et al., 1997). Under physiological conditions, according to the K_{dNa} value we have found, most of the BSAO molecules have this type of binding site occupied by Na^+.

By activity measurements, a second type of binding site (that we call type 2) binds cations with lower affinity compared with type 1. According to Z_aZ_b (~ -4.3), nH (~ 2), and pK_a values (~ 6.2), two negatively charged residues, which could be glutamic and/or aspartic acid residues, appear to be involved in the type 2 binding site.

The results reported above are quite different with respect to those found for PKAO (Padiglia et al., 2001). In fact, these authors reported that Li^+ and Na^+ ions should act as weak noncompetitive inhibitors binding to an empty pocket close to the copper ion. The reversible interaction of these small monovalent cations (Padiglia et al., 2001) and of NH_4^+ and Cs^+ in the case of *E. coli* and *H. polymorpha* (Parsons et al., 1995; Plastino et al., 1999) with the carboxylic groups of aspartate (the catalytic base of amine oxidases) or with glutamate residues in this pocket should change the orientation of the TPQ cofactor. Under these conditions, TPQ would be still available for substrate binding but no longer able to carry out a complete catalytic cycle. Conversely, we found that Ca^{2+} and Mg^{2+} and only the monovalent ions with larger ionic radius (such as Cs^+) may bind reversibly to an active-site region of BSAO. This region appears relatively far from the TPQ cofactor (~ 10–12 Å) because it is involved in the interaction with the positive charge in position 10 (from the reactive amino group) of physiological polyamines such as spermine and spermidine. The reversible interaction of the cations with this binding area affects the docking of spermine and spermidine, which represents the first step of the catalytic cycle, but does not interfere with the binding and catalysis of benzylamine and nonylamine. Probably these substrates do not interact with this cation-binding region, being too short (benzylamine) with respect to spermine or interacting with a hydrophobic region (nonylamine). Therefore, the behavior of BSAO in the presence of cations appears quite different with respect to that observed in pig kidney and yeast amine oxidases (Padiglia et al., 2001; Plastino et al., 1999).

According to the K_{dM} values characterizing this type of binding site, the binding of cations of groups IA and IIA should not have a particular physiological relevance. However, the knowledge that the inhibition of amine oxidase activity toward physiological amines such as SPM and SPD

occurs when this site is occupied by positively charged species may help to design amine oxidase inhibitors, which are the object of intensive research with pharmacological application (Artico et al., 1988; Yu et al., 2001).

APPENDIX

The Na^+ relaxation data were fitted by Eq. 5 (see text), obtained assuming the presence of only one type of Na^+-binding site on BSAO (E), characterized by the dissociation constant K_d. In particular, on the basis of the following equilibrium:

$$E + Na^+ \overset{K_{dNa}}{\rightleftharpoons} E\text{-}Na^+,$$
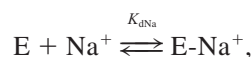
the dissociation constant of the $E\text{-}(Na^+)_n$ complex is:

$$K_{dNa} = ([E][Na^+])/[E\text{-}Na^+] \qquad (7)$$

The mass balances for Na^+ and E are:

$$[Na^+]_0 = [Na^+] + [E\text{-}Na^+] \qquad (8)$$

and

$$[E]_0 = [E] + [E\text{-}Na^+], \qquad (9)$$

where $[Na^+]_0$ and $[E]_0$ are the analytical concentrations of Na^+ and BSAO, respectively.

Because under our experimental conditions $[Na^+]_0 \approx [E]_0$ in Eq. 8, the result is $[Na^+] \approx [Na^+]_0$, and we can write:
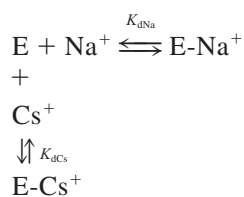
$$[E\text{-}Na^+] = ([E]_0 \times [Na^+]_0)/(K_{dNa} + [Na^+]_0) \qquad (10)$$

Consequently, in accordance with Eq. 4 (see text), it is

$$R_{Na} = \{[E]_0/(K_{dNa} + [Na^+])\} \times R_{Na\text{-}E}, \quad \text{(Eq. 5 in the text)}$$

where $R_{Na\text{-}E}$ is the relaxation rate of the Na^+ bound to the enzyme.

On the basis of the results obtained with Na^+, for Cs^+ ion we also assume the presence of one type of binding site and take into account the competition between Cs^+ and Na^+ for this site, according to the scheme:

$$
\begin{array}{l}
E + Na^+ \overset{K_{dNa}}{\rightleftharpoons} E\text{-}Na^+ \\
+ \\
Cs^+ \\
\updownarrow K_{dCs} \\
E\text{-}Cs^+
\end{array}
$$

where K_{dNa} and K_{dCs} are the dissociation constants of the $E\text{-}Na^+$ and $E\text{-}Cs^+$ complexes, respectively.

The mass balances are:

$$[E]_0 = [E] + [E\text{-}Na^+] + [E\text{-}Cs^+] \qquad (11)$$

$$[Na^+]_0 = [Na^+] + [E\text{-}Na^+] \approx [Na^+] \qquad (12)$$

$$[Cs^+]_0 = [Cs^+] + [E\text{-}Cs^+] \qquad (13)$$

The experimental relaxation rate of $^{133}Cs^+$ nucleus is given by:

$$R = [(1/T_2)_{+BSAO} - (1/T_2)_{-BSAO}] = \chi_B R_B - \chi_F R_F, \quad (14)$$

where $(1/T_2)_{+BSAO}$ and $(1/T_2)_{-BSAO}$ are the relaxation rates of the Cs^+ in the presence and in the absence of BSAO, χ_F and χ_B are the molar fraction,

and R_B and R_F are the relaxation rate of the ion bound to the protein (B) and free ion (F), respectively. As a result and according to scheme above, the following equation was obtained (Eq. 6 in the text):

$$R_{Cs} = A + [E]_0 + [Cs^+]_0 + \{(A + [E]_0 + [Cs^+]_0])^2$$
$$- 4[E]_0[Cs^+]_0\}^{1/2}\{R_{E\text{-}Cs}/(2[Cs^+]_0)\},$$

where $[Cs^+]_0$ is the analytical cesium concentration, $A = K_{dCs}$ $(1 + [Na^+]_0/K_{dNa})$. A value of $K_{dNa} = 54$ mM was used to fit the experimental data.

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