Electrostatic control of oxidative deamination catalysed by bovine serum amine oxidase

Roberto STEVANATO,* Bruno MONDOVI,† Olivia BEFANI,† Marina SCARPA‡ and Adelio RIGO§||

*Department of Physical Chemistry, University of Venice, Venice, Italy, †Department of Biochemical Sciences and Center of Molecular Biology, University of Rome 'La Sapienza', Rome, Italy, ‡Department of Physics, University of Trento, Trento, Italy, and §Department of Biological Chemistry, University of Padua, Padua, Italy.

The ionic-strength-dependence of steady-state kinetic parameters (k_c and K_m') for non-biogenic (benzylamine, butylamine) and biogenic (spermine, spermidine) amines has been measured in the bovine serum amine oxidase reaction. The catalytic rate constant (k_c) values are similar (0.9–2.5 s^{-1}) for all the substrates studied and are almost constant over the experimental ionic strength range (24–155 mM). In contrast, K_m' values are in the range 6–2300 μM and undergo a 4–12-fold increase with increasing ionic strength, parallelled by a decrease in catalytic efficiency. From an analysis of the k_c and K_m' values and their dependence on ionic strength, we conclude that more than one negative site is involved in the binding of these amines and that the relative dielectric constant of the binding site is lower than that of aqueous solutions.

INTRODUCTION

Bovine serum amine oxidase (amine–O_2 oxidoreductase, deaminating; EC 1.4.3.6) is a mammalian copper enzyme which catalyses the oxidative deamination of primary amines, according to the following equation [1]:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2 \quad (1)$$

This enzyme shows the highest activity towards spermine (SPM) and spermidine (SPD) [1,2], which together with putrescine are normal constituents of blood.

Bovine serum amine oxidase, a dimer of 170 kDa, has been shown to contain Cu(II) and an organic cofactor. The structure of the organic cofactor has been recently reinvestigated [3] and 6-hydroxydopa has been proposed [4] instead of pyrroloquinoline quinone [5] or pyridoxal phosphate [6,7]. The common characteristic of these prosthetic groups is the presence of at least one carbonyl group, which has been demonstrated to be directly involved in the formation of the activated complex [1]. In fact, reaction (1) can be formalized as a sequence of partial reactions involving: (i) formation of an imine (Schiff base) between the amine and the cofactor with the parallel reduction of the enzyme, (ii) hydrolysis of the imine; (iii) enzymic reoxidation by O_2 with formation of H_2O_2 [1]. Klinman and co-workers [8–10] reported a deuterium isotope effect on k_c and k_c/K_m' parameters for benzylamine (BZA) and similar monoamines, indicating that C-H bond cleavage is partially rate-limiting for these parameters.

In the present studies, as in most of the kinetic and mechanism studies reported on this enzyme, BZA has been utilized, because its oxidation can be conveniently followed by u.v. spectroscopy at 250 nm. As BZA is a monoamine and at low concentration is a poor substrate for bovine serum amine oxidase [2], any reaction mechanism hypothesis based on BZA kinetic data should be correlated with natural substrates, to avoid misleading interpretation of the oxidative deamination of polyamines. In fact, at physiological pH, SPD and SPM are protonated and possess three and four charges respectively [11,12]. The polycationic character of these biomolecules makes them suitable to interact strongly with the electrical charge present around or in the active site of the enzyme. This electrostatic interaction between the positively charged substrate and the enzyme is usually not taken into account in mechanistic studies of this class of enzymes, apart from some data on specific effects of salts on amine oxidase activity [13–15]. In fact, most of the studies indicate the nucleophilic attack of the unprotonated amine on the carbonylic group of the cofactor as the first step of the reaction. In the present study, we have focused on the effect of ionic strength (I) on the activity of bovine serum amine oxidase toward biogenic (SPM and SPD) and non-biogenic [BZA and butylamine (BUA)] substrates in an effort to determine the effect of electrostatic interactions on catalytic parameters.

According to the Debye–Hückel theory, the relationship between the activity coefficient, ¥, of a reacting ion, i, of charge Z_i and I is given, for dilute solution, by

$$\log ¥ = -CZ_i^2\sqrt{I}$$

where

$$C = 1.82 \times 10^6 \sqrt{(\rho/\epsilon^3 T^3)} \quad (2)$$

where ρ is the density, ϵ is the dielectric constant of the reaction medium and T is the absolute temperature. C is 0.523 $M^{-\frac{1}{2}}$ for the reaction occurring in water at 37 °C [16].

The variation of the kinetic rate constant (or equilibrium constant) with ionic strength depends on the way in which the activity coefficient ¥ of the species involved in the process varies with I. This leads, in the case of the kinetic rate constant, to the following equation:

$$\log k = \log k_o + 2CZ_AZ_B\sqrt{I} \quad (3)$$

where Z_A and Z_B are the charges of the species involved in the formation of the activated complex and k_o is the kinetic rate constant at $I = 0$ [17].

EXPERIMENTAL

The chemicals were analytical grade. Purified bovine serum amine oxidase was prepared from bovine blood as described by Mondovi et al. [18].

Initial-velocity studies were carried out by monitoring the

Abbreviations used: SPM, spermine; SPD, spermidine; BZA, benzylamine; BUA, butylamine; I, ionic strength.

|| To whom correspondence should be addressed: Dipartimento di Chimica Biologica, via Trieste 75, 35121 Padova, Italy.

increase in absorbance at 555 nm for H_2O_2 formation [see eqn. (1)] by a previously described [19] spectrophotometric method, requiring peroxidase and a reduced dye. An absorption coefficient of 1.2×10^4 $M^{-1} \cdot cm^{-1}$ was taken for the calculation of the amount of H_2O_2 produced. Under our experimental conditions, the increase in absorbance at 555 nm was always proportional to the rate of polyamine oxidation. The kinetic runs were recorded on a Beckman DU 7 u.v.–visible spectrophotometer.

First-order (k_c) and second-order (k_c/K_m') rate constants were calculated from apparent $V_{max.}$ and $V_{max.}/K_m'$ values by assuming a subunit M_r of 85000 [8].

Unless otherwise indicated, all measurements were carried out in solutions equilibrated in air, with the same batch of enzyme, characterized by a specific activity of 0.23 unit/mg. The enzyme unit was defined, according to Tabor et al. [20], as the amount of enzyme catalysing an increase in absorbance at 250 nm of 0.001/min when BZA was the substrate. If not otherwise stated, 7.5 mM Mes and 15 mM Tris, pH 7.20, were used as buffer. The ionic strength (defined as $I = 0.5 \Sigma M_i Z_i^2$, where M_i is the molarity of the ion i of charge Z_i) was adjusted by addition of NaCl. Initial rates were expressed in terms of H_2O_2 produced ($\mu M \cdot s^{-1}$).

RESULTS

Effect of various ions on enzyme activity

The effect of various ions on bovine serum amine oxidase activity was tested utilizing SPM as substrate. From these measurements, carried out under conditions close to physiological, i.e. $[O_2] = 0.216$ mM ($pO_2 = 21.2$ kPa), [SPM] = 20 μM, pH 7.0 and 37 °C, it appeared that: (i) the initial rate of oxidative deamination was highly sensitive to the total salt concentration, i.e. I; (ii) there were no specific effects of the ions tested (Na^+, K^+, Li^+, Cl^-, ClO_4^-, F^-) on the oxidation rate. In fact the same initial-rate values were observed when different salts were used to obtain a given I value; see Figure 1, where the logarithm of the initial rate versus $\sqrt{I}$ is reported, as suggested by the Debye–Hückel theory [21].

Steady-state kinetic parameters

The initial rates of oxidative deamination of SPM, SPD, BZA and BUA by bovine serum amine oxidase were measured at 37 °C and I 155 mM, the O_2 concentrations being in the range 30 μM–1.1 mM ($pO_2 = 3$–101 kPa). As under these experimental conditions the reaction rates were similar within experimental error, the K_m values for O_2 measured in the presence of various amines should be $\leqslant 10$ μM. This limiting value, in the case of BZA, is in accordance with the value of $K_m' = 13.5$ μM obtained at 25 °C in 0.1 M potassium phosphate buffer, pH 7.2, reported by Oi et al. [22]. According to these findings, the kinetic parameters for the various amines were calculated from measurements carried out in air-equilibrated solutions, i.e. under saturating O_2 concentrations. Therefore, as the kinetic evidence available for amine oxidases [14,22] indicated that the oxidative deamination occurs according to a Ping Pong Bi Ter mechanism (the first product is released before the second substrate binds), the dependence of the reaction rate on the substrate concentration, under our experimental conditions, was analysed according to the following equation:

$$v = k_c[E_o]/\{(1 + K_m'/[A]\} \quad (4)$$

where v is the initial rate, A is the amine substrate and $[E_o]$ the total enzyme concentration. According to eqn. (4), the plots $1/v$ versus $1/[A]$ were straight lines (experimental points $n \geqslant 8$, $r \geqslant 0.995$).

Dependence of k_c and K_m' on I and temperature

The kinetic parameters k_c and K_m' were calculated for SPM, SPD, BUA and BZA, according to eqn. (4) from measurements carried out at 37 °C under saturating conditions for O_2 and at various I values in the range 24–155 mM. The linear dependence of log k_c and log K_m' on $\sqrt{I}$ is given in Figure 2. All the plots were linear up to at least $I = 160$ mM. In some cases, a deviation

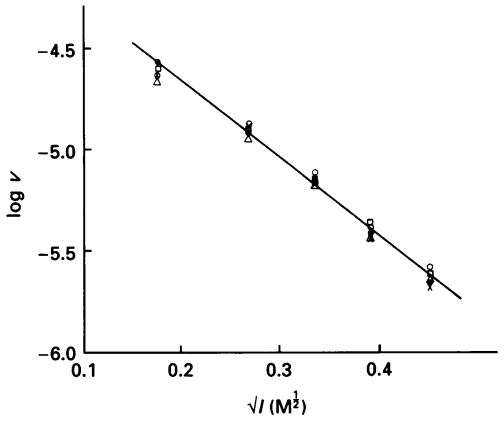

Figure 1 Dependence of bovine serum amine oxidase activity on increasing I by addition of various salts

The salts utilized were KCl (○), NaCl (□), KF (△), $NaClO_4$ (●) and LiCl (×). The experiments were carried out in 5 mM potassium phosphate buffer, pH 7.0, containing 1.5 mM 4-aminophenazone, 2 mM *NN*-dimethylaniline, 20 μM SPM, 1 μM horseradish peroxidase and 4.7 μM bovine serum amine oxidase. The temperature was 37 °C. The activity is expressed as initial rate ($M \cdot s^{-1}$).

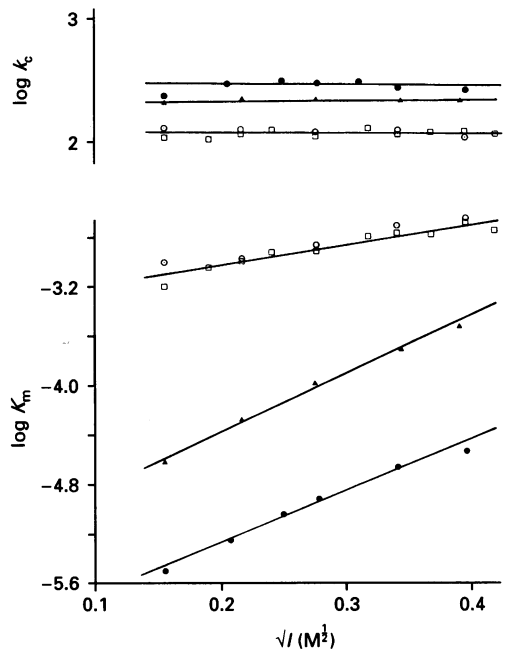

Figure 2 Dependence of k_c and K_m' on ionic strength for SPM (●), SPD (▲), BZA (□) and BUA (○) in the bovine serum amine oxidase reaction

Experimental conditions were as in Figure 1 except that the concentration of bovine serum amine oxidase was 1.2 μM and the buffer system was 15 mM Tris and 7.5 mM Mes, pH 7.20. The k_c and K_m units are s^{-1} and M respectively.

Table 1 $2CZ_AZ_B$ and Z_B values for the oxidative deamination of various amines

These values have been calculated from the plots of Figure 2. Z_B was calculated according to eqn. (3) assuming $C = 0.52\ M^{-\frac{1}{2}}$.

	$\lvert 2CZ_AZ_B \rvert$ ($M^{-\frac{1}{2}}$)		Z_B
	From the k_c values	From the K_m' values	active-site charge
SPM	0	4.2	−1.1
SPD	0.05	4.6	−1.5
BZA	0	1.9	−1.9
BUA	0	1.9	−1.9

Table 2 Steady-state kinetic parameters for the oxidative deamination of SPM, SPD, BZA and BUA catalysed by bovine serum amine oxidase, measured at 37 °C and at two *I* values

Experimental conditions were as in Figure 2.

	I = 155 mM		I = 45 mM	
Substrate	K_{m_A} (μM)	k_c (s^{-1})	K_{m_A} (μM)	k_c (s^{-2})
SPM	30	2.32	5.3	2.46
SPD	310	1.90	53.3	1.84
BZA	2260	1.08	1030	0.97
BUA	2310	0.93	1090	1.03

from linearity was observed at higher I values. This deviation may be due to the limits of the Debye–Hückel theory at high I values [21] and/or inactivation of the enzyme at I values exceeding the physiological conditions as proposed by Bardsley et al. [14] for pig kidney amine oxidase.

According to eqn. (3), the values of the product $2CZ_AZ_B$ were calculated from the slopes of plots of log k_c versus $\sqrt{I}$ and log K_m' versus $\sqrt{I}$, and their absolute values are reported in Table 1.

From Figure 2, it appears that the k_c values are independent of I and slightly dependent on amine structure. In contrast, the K_m' values are very sensitive to these two parameters. In fact the K_m' values increase by more than two orders of magnitude on passing from SPM to BUA, at $I \approx 80$ mM. Furthermore, the K_m' values for SPM and SPD appear to be more sensitive to I ($\lvert 2CZ_AZ_B \rvert \approx 4$–5) than those for BZA and BUA ($\lvert 2CZ_AZ_B \rvert \approx 2$). Because of the high value of $2CZ_AZ_B$ in the case of SPM and SPD, the K_m' values for these substrates increase by more than an order of magnitude when I is increased from 24 to 155 mM. As a consequence, the catalytic efficiency, k_c/K_m', of bovine serum amine oxidase towards various amines is dominated by the $1/K_m'$ factor.

In Table 2, the values of the kinetic parameters k_c and K_m' for various amines, measured at two ionic strengths, are reported.

DISCUSSION

From an inspection of the data collected in Table 1 and in Figures 1 and 2, the following facts emerge clearly: (i) the effect of the different ions tested is due only to electrostatic interactions. In fact, enzyme activity is affected by I and not by the type of salt utilized (NaCl, $NaClO_4$, KF, KCl, LiCl) (Figure 1); (ii) the electrostatic interactions between the positively charged amine and the charges around or in the active site of bovine serum amine oxidase dominate the K_m'; (iii) k_c values are not affected by the electrostatic interactions and very little by the substrate structure (Figure 2).

As under our experimental conditions the overall reaction rate is not affected by O_2 concentration, we should clarify the practical independence of the k_c values of the type of amine and I and the strong dependence of the K_m' values on these parameters. To this purpose we have taken into consideration the possible critical steps of the oxidative deamination process. These steps appear to be: (1) formation of a molecular complex between the enzyme and the amine; (2) a condensation reaction involving the generation of an imine (Schiff base); (3) imine isomerization involving C-H bond cleavage and the parallel reduction of the enzyme; (4) hydrolysis of the isomerized imine; (5) oxidation of the enzyme by O_2. On account of the known chemical mechanisms of these steps, direct interaction of charges may occur only in steps 1 and 2. With regard to step 2, the formation of Schiff base, it should involve a nucleophilic attack by the unprotonated nitrogen of the amine on the electron-deficient carbonyl group of the cofactor [23]. This attack must be preceded by dissociation of the protonated amine in the active site [24]. Then, in step 2, charge interactions take place if the dissociation process occurs in the active site and is assisted by negatively charged groups. However, on account of the large differences in pK_a of BZA and BUA (9.33 and 10.77 respectively), the very close values found for k_c of these amines and their independence of I seem to exclude step 2 as the rate-determining step.

On account of these considerations, we may conclude that steps 3–5, not involving direct charge interactions, control the k_c value, at least partially. The strong dependence of the apparent Michaelis–Menten constant on I, and the independence of k_c of I, indicate that electrostatic interactions control only the initial step of the reaction. In fact, if $K_m' = (k_c + k_2)/k_1$, where k_1 and k_2 are the kinetic rate constants of the formation and dissociation of enzyme–substrate complex respectively, the plot of log k_c/K_m' versus $\sqrt{I}$ gives the dependence of k_1 on I. This conclusion was reached on the basis of the following considerations: $k_c/K_m' = k_1$ if $k_c \gg k_2$, whereas $k_c/K_m' = k_c k_1/k_2$ if $k_c \ll k_2$. In the latter case, as k_c and k_2 are independent of I, the dependence of the ratio k_c/K_m' on I is that of k_1 on I. Therefore, on the basis of the slopes of the plots k_c/K_m' versus $\sqrt{I}$, obtained from the data of Figure 2, it appears that the positively charged amines interact with negative charges localized near or in the active site.

The average value of the active-site charge, Z_B, has been calculated assuming that the amines we have studied are fully protonated at pH 7.2 [11,12] and taking $C = 0.523\ M^{-\frac{1}{2}}$, i.e. the value for a reaction occurring in water at 37 °C. The calculated values are reported in the last column of Table 1. From these values it appears that the active-site negative charge, 'seen' by the amine, decreases with increasing amine charge. This conclusion appears unrealistic. Furthermore, it is difficult to accept that a single-charged amine, such as BZA and BUA, can interact simultaneously with two negative single charges, which could be identified with charged amino acid residues. As in the case of BZA and BUA a single negative site should be involved in their binding, the high Z_AZ_B value we have calculated instead of the expected −1 can be explained if we consider that the dielectric constant of the active site is lower than that of an aqueous solution. In particular if we assume $Z_AZ_B = -1$ for BZA and BUA, C is 0.95 and, according to eqn. (2), a dieletric constant value of 50 can be calculated for the active site. Assuming $\epsilon = 50$,

$Z_A Z_B \approx -2.3$ was calculated for SPM and SPD, indicating that more than a single negative site is involved in the binding of the polyamines. These values should be taken into qualitative consideration, as they depend on the spatial distribution of the charges of the polyamine and structure of the active site. In fact, the electrostatic interactions between charged molecules are regulated by their average electrical fields which depend on their molecular structure. These results show the complexity of oxidative deamination and the need for more studies on the nature of the active site.

This work was partly supported by the Special Project on Biotechnology and Bioinstrumentation and by grant no. 90.03097. CT04 from the Consiglio Nazionale delle Ricerche.

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Received 9 August 1993/26 October 1993; accepted 28 October 1993