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Influence of Electrostatic Interactions and Hydrogen Bonding on the Activity of Cyclodextrin-based Superoxide Dismutase Models

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The influence of electrostatic interactions and hydrogen bonding between the substrate and the active site in superoxide dismutase (SOD) models based on Cu(II) complexes of cyclodextrin dithiocarbamates is studied. Polar and positively charged groups present in the structure of the complexes were found to increase its activity in 35–70%. Our studies reveal the importance of these two features in enhancing the activity of SOD model complexes.

Keywords: Cyclodextrin; Dithiocarbamate; SOD-like activity; Copper complex

INTRODUCTION

Copper,Zinc-superoxide dismutase (Cu,Zn-SOD) [1] is an extremely high efficient metalloenzyme ($k = 2 \times 10^9\,\mathrm{mol^{-1}\,L\,s^{-1}}$) in its dismutation of superoxide radical:

$$2O_2^{\bullet -} + 2H^+ = H_2O_2 + O_2$$

This overall reaction involves a cyclic two-step process in which the Cu(II) center is consecutively reduced and reoxidized to form the products. However, beyond this apparently simple mechanism, several structural and kinetic features are manifested, resulting in a nearly perfect natural catalytic system. For example, the residue of Arg-141, located in the proximity of the active site [2] is assumed to play two important roles. Its positive charge electrostatically attracts the substrate toward the active site and, once superoxide radical is coordinated to Cu(II), it stabilizes it through a H-bond formation with the guanidine residue [3]. Theoretical studies [4] as well as chemical modification of Arg-141 [5] have demonstrated that the positive charge of Arg-141 is responsible for the enhancement of the activity in the wild metalloenzyme in about 30–90%.

Modeling enzyme-substrate interactions with artificial systems is a great challenge for chemists and has been the subject of extensive study [6]. There are several reports on Cu(II) complexes with SOD-like activity [7] but systematic

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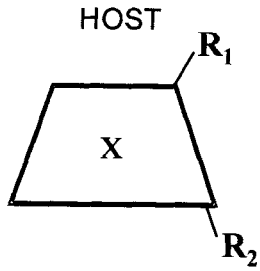
studies of the enzyme-substrate recognition events in Cu,Zn-SOD using models are scarce or rather absent [8].

Cyclodextrins (CD) [9], cyclic oligomers composed of α-(1 $\rightarrow$ 4)-linked D-glucopyranose units, and its derivatives have been widely employed as recognition centers in artificial enzymes [10]. They possess a central hydrophobic cavity and hydroxyl groups located at the two rims of the cavity that allow them to form stable inclusion complexes with a wide variety of guests [11]. In the present work we have made use of the main structural characteristics of CDs to model separately the electrostatic and H-bond interactions in Cu,Zn-SOD.



x = α; R_1 = OH; R_2 = $N(CH_3)CSS^-$: **αC2DTC**
x = β; R_1 = OH; R_2 = $N(CH_3)CSS^-$: **βC2DTC**
x = β; R_1 = $N(CH_3)CSS^-$; R_2 = OH: **βC6DTC**

GUEST

RNX_3^+

R = 4-$CH_3C_6H_4$; X = CH_3: **TTMA**
R = 4-$CH_3C_6H_4$; X = C_2H_5: **TTEA**
R = C_6H_{11}; X = CH_3: **CTMA**
R = C_6H_{11}; X = C_2H_5: **CTEA**

FIGURE 1 Structure of cyclodextrin derivatives and QAS studied.

RESULTS AND DISCUSSION

Modeling the Electrostatic Interactions in Cu,Zn-SOD

According to the classical mechanism proposed by Trainer *et al.* [3] in the first stage of the reaction superoxide radical is electrostatically attracted towards the active site and coordinates to the Cu(II) center. To model this process we have selected the inclusion complexes of cyclodextrin dithiocarbamates with quaternary ammonium salts (QAS) having hydrophobic residues (Fig. 1). Dithiocarbamate moieties were selected because they form highly stable neutral Cu(II) complexes of general formula CuL_2, avoiding the presence of free copper or unsaturated species under the high dilution conditions employed in the activity determination. Moreover, as neutral compounds their activity is independent of pH and ionic strength.

The SOD-like activity is a function of several factors. Among them the structural factor is very important and the dependence of the SOD-like activity with the geometry of the coordination sphere of the Cu(II) center is well documented [12]. Distortions from square planar geometry help to reduce the steric barrier associated to the change in coordination sphere related to the catalytic process. EPR has been shown to be a valuable tool to detect any distortion from the square-planar geometry of Cu(II) centers [7b, 13]. In order to detect any possible effect of the inclusion of the QAS on the geometry of the corresponding copper(II) complex, their EPR spectra in aqueous solution were recorded for a QAS:complex: molar ratio of 100:1 at 300 and 100 K. At room temperature, a significant line broadening was observed upon addition of the guests, suggesting inclusion complex formation. The EPR parameters of the Cu(II) inclusion complexes recorded in frozen solution at 100 K were very similar, within the experimental error, to the spectrum of the corresponding Cu(II) complex without the included QAS. This indicates that the geometry of the complex is not affected by the inclusion of the QAS into the CD cavity.

In the presence of QAS at guest:host molar ratios ranging from 1 to 200, Cu(βC2DTC)$_2$,

$Cu(\beta C6DTC)_2$ and $Cu(\alpha C2DTC)_2$ showed an increase in their SOD-like activity in 35–76% with a saturation behavior when compared with the complexes without guest. A similar procedure as that already reported by us [8a] was used to determine the apparent binding constants K of the systems QAS/$Cu(\alpha C2DTC)_2$, from the variations of the SOD-like activity as a function of the QAS:complex molar ratio. The following values of K (in $mol^{-1}dm^3$), assuming the formation of a 1:1 inclusion complex, were obtained: 410 (CTMA), 260 (CTEA), 3600 (TTMA) and 3900 $mol^{-1}dm^3$ (TTEA). The differences in these values are attributed to the smaller size of the hydrophobic moiety of TTMA and TTEA, which fit perfectly into the small cavity of α-CD, with respect to CTMA and CTEA. In fact, a ^{1}H-NMR study on the inclusion of TTMA and CTMA in the cavity of native α-CD showed that TTMA induces a higher shift in the inner protons H-3 (−0.11 ppm) and H-5 (+0.13 ppm) than CTMA (−0.01 ppm and +0.02 ppm respectively). Therefore, $Cu(\alpha C2DTC)_2$ is more selective to p-tolyl QAS and forms a more stable inclusion complex with these guests, an opposite behavior to that found with βCD containing complexes [8].

Since the observed increase in the SOD-like activity can not be attributed to the geometric factor, we interpret this observation considering that the QAS plays the role of a "substrate attractor" by promoting the mutual electrostatic interaction between substrate and artificial enzyme.

Now, if the observed increase in the SOD-like activity of the included copper(II) complexes is actually due to the electrostatic attraction that the included QAS could exert over the substrate, this activity is expected to be affected by the ionic strength of the medium. An increase in the ionic strength should reduce the effect of such attraction due mainly to a partial neutralization of the positive charge of the QAS. The corresponding experiments were carried out with $Cu(\beta C2DTC)_2$ and $Cu(\beta C6DTC)_2$ in the presence of TTMA and CTMA (Fig. 2). While the activity of the $Cu(\beta C2DTC)_2$ + TTMA inclusion complex is only slightly affected by the variation of the ionic strength of its aqueous solution, the other three inclusion complexes, $Cu(\beta C6DTC)_2$ + CTMA, $Cu(\beta C6DTC)_2$ + TTMA and $Cu(\beta C2DTC)_2$ + CTMA, decreased their activities in this order. According to the electrostatic interpretation given to the increase of the SOD-like activity, it should be expected that the effect of the ionic strength depends on the stability of the formed inclusion complex and on the net positive charge on the nitrogen atom of the ammonium group. The reported binding constants are: 910 ($Cu(\beta C6DTC)_2$ + CTMA), 1900 ($Cu(\beta C6DTC)_2$ + TTMA), 2000 ($Cu(\beta C2DTC)_2$ + CTMA) and 750 $mol^{-1}dm^3$ ($Cu(\beta C2DTC)_2$ + TTMA); and the calculated net charges on the nitrogen atoms of the ammonium groups are: 0.0854 (CTMA) and 0.0288 (TTMA) [8a]. The inclusion complex not affected by the ionic strength, $Cu(\beta C2DTC)_2$ + TTMA, is the less stable and its QAS presents the less positively charged nitrogen atom. On the contrary, the inclusion complex most affected by the ionic strength, $Cu(\beta C2DTC)_2$ + CTMA, is characterized by the highest inclusion constant and its

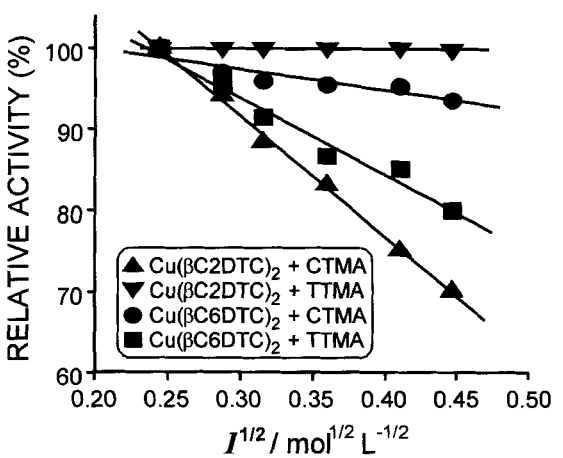


FIGURE 2 Effect of the ionic strength (I) on the SOD-like activity of different inclusion complexes at a QAS:complex molar ratio of 100:1 in 10 mM phosphate buffer (pH 7.8) at 298 K.

QAS contains the highest positive charge density on the nitrogen atom. These results confirm the electrostatic interpretation given here to the increment of the SOD-like activity in the presence of the guests.

Similarly to our model, in the wild enzyme the activity decreases with the ionic strength [14]. However, in this case the effect of the ionic strength on the activity is a combination of several factors: at physiological pH the enzyme total charge equal to −4 repels the substrate but this repulsion is compensated by the local positive electric field generated by the Cu(II) and Zn(II) ions and positively charged amino-acid residues that attract it. In the presence of salts, the second factor becomes more important and, as a result, the activity decreases as the ionic strength increases [4]. It is clear from our model that this experiment only involves the *local* factor allowing a direct measurement of the electrostatic contribution to the activity in the vicinity of the active site.

Modeling H-bonding in Cu,Zn-SOD

To model this feature, we selected a series of complexes with dithiocarbamate ligands containing and not-containing pendant hydroxyl groups and determined their SOD-like activities. The complexes were also characterized by EPR spectroscopy (Tab. I). Figure 3 shows the experimental and simulated EPR spectra of Cu(βC2DTC)$_2$ in water at 100 and 300 K.

TABLE I EPR parameters (frozen solution, 100 K) and SOD-like activity (pIC_{50}) of the studied complexes

Complex	$g_{\|\|}$	$A_{\|\|}$ (cm^{-1})	$A_{\perp}$ (cm^{-1})	f (cm)[a]	pIC_{50}
Cu(αC2DTC)$_2$	2.061	185	41	111	5.61
Cu(βC2DTC)$_2$	2.062	187	41	110	5.34
Cu(βC6DTC)$_2$	2.062	187	41	110	4.22
Cu(PipDTC)$_2$[b]	2.069	191	40	107	4.60
Cu(HMPipDTC)$_2$[b]	2.071	190	41	108	5.11
Cu(HEPipDTC)$_2$[b]	2.071	191	41	108	5.44

[a] $f = g_{\|\|}/A_{\|\|}$.
[b] EPR spectra recorded in 50% DMSO solution.

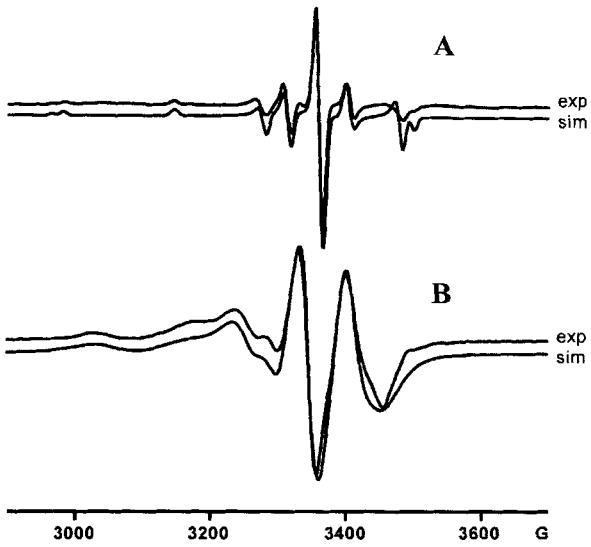


FIGURE 3 Experimental and simulated EPR spectra of a 0.01 M aqueous solution of Cu(βC2DTC)$_2$ at 100 K (A) and 300 K (B).

The possible influence of H-bond formation between the ligand and superoxide radical has been already hypothesized by us after finding that the SOD-like activity of Cu(βC2DTC)$_2$ was more than 10 fold higher than that of Cu(DDTC)$_2$ (DDTC = diethyldithiocarbamate) [8b]. That difference was attributed to the fourteen secondary hydroxyl groups contained in the β-CD moiety able to assist substrate fixing to the active site through H-bond. Structurally it is known that, depending on whether they lay in the primary or secondary face, the —OH groups of CD have a different acidity [15]. Since H-bond strength depends on the donor character of the —OH group involved, which is a function of the acidity, it is expectable that attaching the catalytic group in different positions will modify the activity. As can be seen, the activity of the secondary derivative Cu(βC2DTC)$_2$ is more than one order of magnitude higher than that of Cu(βC6DTC)$_2$. The EPR parameters of the three complexes are very similar, therefore the influence of the geometric feature can be discarded. The observed drop in the activity of Cu(βC6DTC)$_2$ can be interpreted in terms of

the different steric and acid-base character of the hydroxyl groups present on both rims of CD. Those groups located on the primary rim are much less acid and more bulky than the secondary ones and therefore H-bond is expected to be weaker in the first case.

From a structural point of view, it is well known that the annular molecule of CD possesses the smallest diameter at the primary rim, so a certain steric hindrance to the approach of the substrate could also be expected for the primary regioisomers. In order to define the possible effect of this steric difference, we prepared the Cu(II) complex of a secondary dithiocarbamate derivative of α-CD, Cu(αC2DTC)$_2$, considering that the secondary rim of this CD is similar in diameter to the primary rim of β-CD [9]. The SOD activity of Cu(αC2DTC)$_2$ is very similar to that of the β-CD analog. This result indicates that the more acidic secondary hydroxyl groups can be assisting the catalytic process. Therefore, the observed difference in SOD-like activity between Cu(βC2DTC)$_2$ and Cu(βC6DTC)$_2$ should mainly be related to the difference in the acidity of their hydroxyl groups that can be involved in substrate fixation (Fig. 4).

In order to generalize the influence of H-bond formation, a series of Cu(II) dithiocarbamates derived from piperidine was also investigated

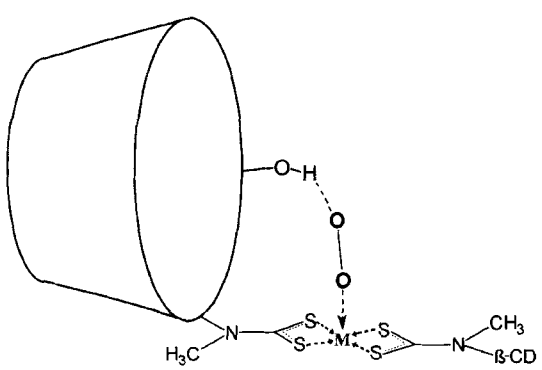
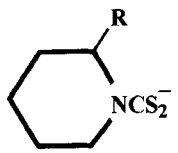


FIGURE 4 Suggested binding of superoxide radical to the active site. The interaction is assisted by the formation of H-bonds with the hydroxyl groups of CD.



R = H: **PipDTC**
R = CH_2OH: **HMPipDTC**
R = CH_2CH_2OH: **HEPipDTC**

FIGURE 5 Structure of piperidine ligands.

(Fig. 5). In this series, Cu(PipDTC)$_2$ presents an axial symmetry according to the EPR spectrum in the solid state ($g_{\parallel} = 2.090$, $g_{\perp} = 2.029$). In the case of the complexes with the hydroxyalkyl chains in position 2, they exhibit EPR spectra in the solid state typical of complexes with a rhombic distortion, with $g_1 = 2.070$, $g_2 = 2.034$ and $g_3 = 2.020$. The analysis of their magnetic parameters suggests a direct interaction of Cu(II) with the hydroxyl group in the apical position. However, in aqueous solution the three EPR spectra are practically identical (Tab. I), indicating that water breaks the interaction of the hydroxyl group with Cu(II), probably through its H-bond formation with water. These three complexes present an extremely planar geometry ($f = 107-108$ cm), so this feature does not affect the analysis. Now, if the hydroxyl groups attached to the alkyl side chains are able to interact in the solid state with Cu(II), it could also be expected that they do so with superoxide radical in aqueous solution through H-bond formation. This would give a consistent interpretation of why both Cu(HMPipDTC)$_2$ and Cu(HEPipDTC)$_2$ attain a SOD-like activity practically one order higher than the non-substituted Cu(PipDTC)$_2$ complex. The analysis of molecular models suggests that the formation of this H-bond is geometrically possible, specially in the case of Cu(HEPipDTC)$_2$, that could be the reason why this complex is the most active of this series.

Our results suggest that the presence of pendant —OH groups in the ligands promotes the mutual interaction between the substrate

and the catalytic center, presumably through the formation of H-bonds. A detailed theoretical study of the formation of H-bonds between superoxide radical and some of these model complexes is in progress in our laboratory.

CONCLUSIONS

The effect of electrostatic interactions and H-bonding in SOD models has been investigated for the first time, demonstrating the importance of these two features in the SOD activity. Although we have analyzed both features separately in the CD complexes, it is most likely that they act cooperatively to catalyze the dismutation of superoxide radical through the formation of a supramolecular structure (Fig. 6). As far as we know, the use of a cyclodextrin inclusion complex to optimize the fixation and accessibility of the substrate in an enzyme model is a novel approach, that differs from the classical strategy consisting in the study of a substrate bounded to the cyclodextrin cavity. In our model the guest molecule also played a catalytic role, allowing us to study a small substrate molecule such as superoxide radical. This is another example of the great versatility of cyclodextrins and the variety of processes that can be studied with these molecules.

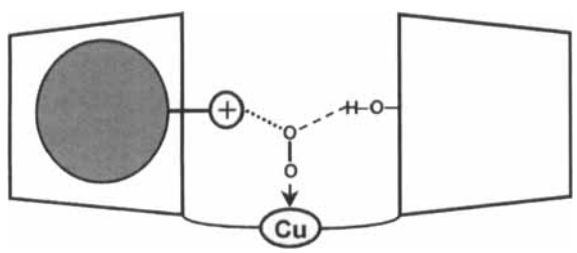


FIGURE 6 Coordination of the substrate at the active center with the formation of a supramolecular compound. The substrate is electrostatically attracted toward the copper center and is stabilized through the formation of an H-bond with a secondary hydroxyl group of the uncomplexed CD moiety.

EXPERIMENTAL SECTION

The β-cyclodextrin derivatives were prepared according to previous reports [8]. The dithiocarbamates were prepared by the reaction of the corresponding amine with CS_2 in the presence of NEt_3 or NaOH at 0°C in a 1:1:1 molar ratio in 70–75% yield and purified by precipitation from acetone or recrystallization from EtOH.

Triethylammonium Mono-2-methylamino-2-deoxy-αCD Dithiocarbamate (αC2DTC)

Mp 181–184°C (dec). UV (H_2O): 256 (24000), 291 (30000). ^{1}H NMR (D_2O), δ 3.20 (s, 3 H, NCH_3), 3.38 (dd, 1 H, H2′), 3.40–3.90 (m, 36 H, H2, H3, H4, H5, H6), 4.87 (d, $^3J_{1',2'} = 1.1$ Hz, 1 H, H1′), 4.91–5.00 (m, 5 H, H1). ^{13}C NMR (D_2O) δ 36.9 (CH_3), 61.7 (C2′), 62.3 (C6), 68.9 (C3′), 72.6, 73.0 (C3, C5), 74.2 (C2), 83.1 (C4), 100.1 (C1′), 103.1 (C1), 207.1 (CSS). FABMS: *m/z*: 1151.9 $[M+H]^+$.

Sodium Piperidyl Dithiocarbamate (PipDTC)

Mp 273–275°C. UV (H_2O): 258 (20000), 290 (27000). ^{1}H NMR (D_2O): δ 1.5–1.8 (m, 6 H, H3, H4), 4.29 (t, 4 H, H2). ^{13}C NMR (D_2O): δ 26.5 (C4), 28.4 (C3), 55.8 (C2), 207.6 (CSS). Anal. Calcd for $C_6H_{10}NS_2Na \cdot 3H_2O$: C, 30.3; H, 6.8; N, 5.9; S, 27.0. Found C, 30.4; H, 7.0; N, 5.7; S, 27.3.

Sodium (±)-2-hydroxymethyl-piperidyl Dithiocarbamate (HMPipDTC)

Mp 226–228°C. UV (H_2O): 259 (21000), 291 (28000). ^{1}H NMR (D_2O): δ 1.3–1.9 (m, 6 H, H3, H3′, H4), 3.07 (t, 1 H, H2′), 3.82 (m, 2 H, CH_2OH), 4.55 (d, 1 H, H2′), 6.09 (t, 1 H, H2). ^{13}C NMR (D_2O) δ 21.0 (C4), 27.4, 27.8 (C3, C3′), 49.8 (C2′), 61.7 (C2), 62.4 (CH_2OH), 207.9 (CSS). Anal. Calcd for $C_7H_{13}NOS_2Na \cdot 3H_2O$: C, 31.3; H, 7.1; N, 5.2; S 23.8. Found: C, 31.4; H, 7.0; N, 5.4; S, 23.5.

Sodium (±)-2-(2-hydroxyethyl)-piperidyl Dithiocarbamate (HEPipDTC)

Mp 146–148°C. UV (H_2O) 256 (22000), 289 (31000). ^1H NMR (D_2O): δ 1.3–1.9 (m, 7 H, H3, H3′, H4, CH_2), 2.19 (m, 1 H $CH_{2'}$), 3.05 (t, 1 H, H2′), 3.57 (m, 2 H, CH_2OH), 4.56 (d, 1 H, H2′), 6.08 (s, 1 H, H2). ^{13}C NMR (D_2O) δ 26.6, 27.7 (C4, C3′), 34.2 (C3), 41.1 (CH_2), 48.5 (C2′), 55.7 (C2), 61.4 (CH_2OH), 207.9 (CSS). Anal. Calcd for $C_8H_{15}NOS_2Na \cdot 3H_2O$: C, 34.0; H, 7.5; N, 5.0; S, 22.7. Found C, 34.2; H, 7.2; N, 5.4; S, 22.5.

The SOD-like activity of the complexes was determined under similar conditions to those reported by Fridovich [16] and with the same specifications as reported elsewhere [8]. All the complexes were formed *in situ* at a 10^{-2}M concentration in aqueous or 50% aqueous DMSO (piperidine complexes). The variations in the SOD-like activity in the presence of different amounts of QAS were determined by fixing the concentration of the complex at its IC_{50} value, while the concentration of the guest were 1–200 fold greater. The binding constants (K) for the systems QAS/Cu-complex were calculated considering the formation of a 1:1 inclusion complex, using the same mathematical procedure already reported [8a].

The influence of the ionic strength (I) on the SOD-like activity was determined for the complexes Cu(βC6DTC)$_2$ and Cu(βC2DTC)$_2$ in the presence of TTMA and CTMA for a QAS:complex molar ratio equal to 100. The concentration of the complex was fixed at its IC_{50} value. The ionic strength of the buffer employed is 0.06 M, while the physiological ionic strength is *ca.* 0.15 M. The study was performed in the range $0.06\,M \leq I \leq 0.20\,M$. The ionic strength was adjusted by adding aliquots of the appropriate KCl stock solution from zero to the desired value of I and was calculated according to the equation $I = 1/2\Sigma c_i z_i^2$, where c_i were the concentrations of HPO_4^{2-}, $H_2PO_4^-$, Cl^-, Na^+ and K^+ ions and z_i their respective charges.

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