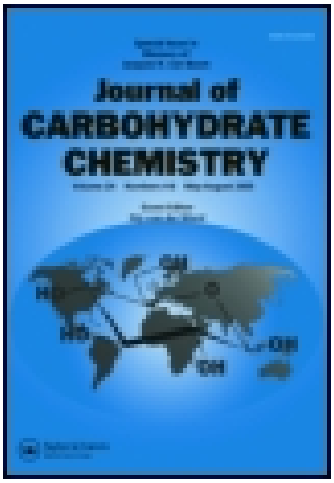

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INFLUENCE OF POSITIVELY-CHARGED GUESTS ON THE SUPEROXIDE DISMUTASE MIMETIC ACTIVITY OF COPPER(II) ß-CYCLODEXTRIN DITHIOCARBAMATES[1]

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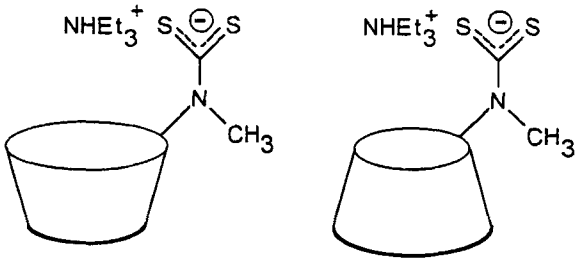
ABSTRACT

The superoxide dismutase (SOD) activity of the Cu(II) complexes of two dithiocarbamate derivatives of cyclodextrin (Cu-C6DTC, Cu-C2DTC), one on the primary (C6DTC) and the other on the secondary (C2DTC) side, was determined in the presence of positively-charged guests which have the general formula RNX_3^+ (where R = C_6H_{11}, X = CH_3 (**3**); R = C_6H_{11}, X = C_2H_5 (**4**); R = *p*-$CH_3C_6H_4$, X = CH_3 (**5**) and R = *p*-$CH_3C_6H_4$, X = C_2H_5 (**6**). The catalytic activity of both Cu-C2DTC and Cu-C6DTC was enhanced 35-70 % by **3-6** for concentrations of the host equivalent to the IC_{50} value of the complex. A correlation was found between the net positive charge of the nitrogen atom and the maximum acceleration attained by both complexes. The formation of an inclusion complex between these cationic guests and the CD moiety of these complexes is proposed to be the determining factor in the observed increase in the catalytic activity.

INTRODUCTION

Cyclodextrins (CDs), cyclic non-reducing oligomers composed of α(1→4)-linked D-glucopyranose units, are a family of naturally occurring molecular receptors with a hydrophobic central cavity in which organic and inorganic molecules can be trapped.[2] The formation of such

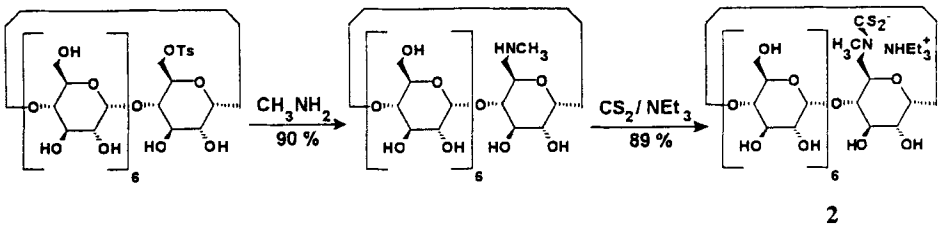
adducts has been extensively studied in recent years due to their potential applications in pharmaceuticals, catalysis, chromatography, design of supramolecular architectures and enzyme mimics.[3] Investigations of several CD derivatives in design of artificial catalysts and enzyme models[4] have contributed to a great extent in the determination of the mechanisms of action of several enzymes. Our interest in superoxide dismutases (SOD), an enzyme that, *in vivo*, disproportionates superoxide radical into oxygen and hydrogen peroxide, has prompted us to construct and study their models.



1 C2DTC **2** C6DTC

We recently reported [5] the synthesis of a model of SOD in which a dithiocarbamate group is appended to the secondary side (2-position) of ß-CD (Cu-C2DTC, **1**). Investigation of SOD mimetic activity of some metal (II) complexes of **1** shows that the highest rate acceleration is exhibited by the Cu(II) complex. We found that the presence of the CD moiety in these complexes aids the superoxide radical ($O_2^{\cdot-}$) dismutation process which is attributed to the ability of the CD moiety to fix the substrate through its secondary hydroxyl groups, provide the catalytic center with a hydrophilic surrounding and promote proton diffusion. There has been considerable interest in the contrasting behavior of CDs bearing substituents attached to the primary (C-6) and the secondary (C-2, C-3) hydroxyl side.[8] They show different binding[9] and catalytic[10,11] properties associated with the capacity to include molecules of variable size and nature. Thus, in this report, we have synthesized the regioisomer of **1** which contains a dithiocarbamate group appended to its primary side (6-position) of ß-CD (Cu-C6DTC, **2**) and compared its activity with **1**.

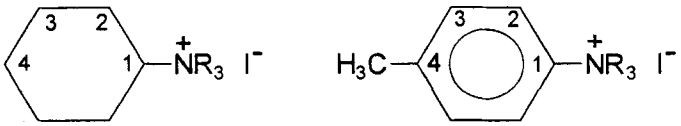
Since the dismutation reaction is not only governed by thermodynamic factors (*i.e.* redox potential of the involved redox couples) but also by kinetic factors (*i.e.* substrate accessibility to the catalytic center), the presence of "substrate attractors" should enhance the catalytic activity. A 2-Å resolution refinement of the crystal structure of the native Cu-Zn superoxide dismutase[6] has revealed that this function is carried out by Arg-141, Lys-120 and Lys-134 which are located



2

Scheme

at the entrance of the cavity and facilitate the $O_2^{\cdot-}$ access to the catalytic center. Since such 'substrate attractors' were absent in our earlier work, we investigated the SOD-like activity of the Cu(II) complexes of isomeric ß-CD dithiocarbamates, **1** (C2DTC) and **2** (C6DTC) in the presence of positively charged guests (**3-6**). The aim of this work is to mimic the function of the above mentioned positively charged amino acid residues as substrate guides and activity enhancers and understand the processes associated with the enzyme-substrate interactions.



3: R = CH_3 **5**: R = CH_3

4: R = CH_2CH_3 **6**: R = CH_2CH_3

RESULTS AND DISCUSSION

Synthesis of Cu-C6DTC

The synthesis of **2** was accomplished as shown in the Scheme. Mono-6-*O*-tosyl-ßCD[14] was reacted with 40% aqueous CH_3NH_2 and resulting product, mono-6-methylamino-6-deoxy-ß-CD, was purified by cation exchange chromatography. This monoamino derivative of CD was reacted with in 2% aq NEt_3 which upon usual workup afforded triethylammonium mono-6-methylamino-6-deoxy-ß-CD-dithiocarbamate (2) in 89 % yield.

Comparison of the SOD-like activity of Cu-C2DTC and Cu-C6DTC

The effect of the substitution pattern of isomeric CD dithiocarbamates on the SOD-like activity of the Cu(II) complexes was analyzed by comparing the catalytic activity of Cu-C6DTC

with that previously reported for Cu-C2DTC.[5] The IC_{50} value of the former was found to be 60 μM, 13 fold less than the latter. Since the thermodynamic properties of the catalytic centers of both complexes are similar, the observed decrease in the catalytic activity is explained in terms of kinetic factors and attributed to the nature of hydroxyl groups located at the two rims of CD. It is known[7] that the hydroxyl groups at the 2-position of CD the most acidic (pK_a=12.2) and those at the 6-position are most basic (pK_a=15-16). We have suggested[5] that the $O_2^{\cdot-}$ interaction with Cu-C2DTC takes place via hydrogen bonding with the secondary hydroxyl groups which promotes proton diffusions. In the case of Cu-C6DTC, although the formation of such hydrogen bonds is possible, the proton transfer step is not favored due to weaker acidity of the primary hydroxyl groups. Alternatively, decrease in the activity can be attributed to the presence of the more bulky hydroxymethyl groups close to the catalytic center which present a steric hindrance to the approach of the substrate. Thus, in our case, the large decrease in the catalytic activity observed for Cu-C6DTC appears to be a consequence of the different steric and acid-base characteristics of the hydroxyl groups that could assist the substrate binding. These results illustrate the importance of cooperative groups in the effectiveness of substituted CDs as enzyme mimics.

The Role of Ions in the SOD Activity

In erythrocyte SOD, an inactivation in 10-20% has been observed[12] after chemical modification of Arg-141 which is considered to play an important role in electrostatic attraction of $O_2^{\cdot-}$ to the active site. Moreover, theoretical calculations of the diffusion of $O_2^{\cdot-}$ to Cu-Zn-SOD show that when Arg-141 is neutralized, the catalytic activity decreases by about 30 %.[13] In the present work, we studied the influence of positive charges on the SOD-like activity of a synthetic model. The SOD-like activity of Cu-complexes of **1** and **2** was determined in the presence of quaternary ammonium salts which have hydrophobic residues and are capable of forming inclusion complexes with the CD moiety of the complexes. The results expressed in terms of the percent of acceleration of $O_2^{\cdot-}$ dismutation relative to non-guest containing assays (RA) are presented in Figure 1. The copper complex concentration used was equivalent to its previously determined IC_{50} value and the guest:host molar ratio ranged from 1 to 200. In all cases the dismutation reaction is accelerated 35-70% in the presence of the guests compared to the complex alone. Thus, our synthetic model mimics the entire real enzyme and these results for the artificial enzyme are in complete agreement with previous reports for the native enzyme. [12,13]

The catalytic activity was also studied in the presence of an anionic guest, *p*-toluene sulphonate ion which also forms an inclusion complex with CD. In this case, as expected, the opposite effect is observed and this guest exhibits a slight inactivation, *ca.* 5%, for both **1** and

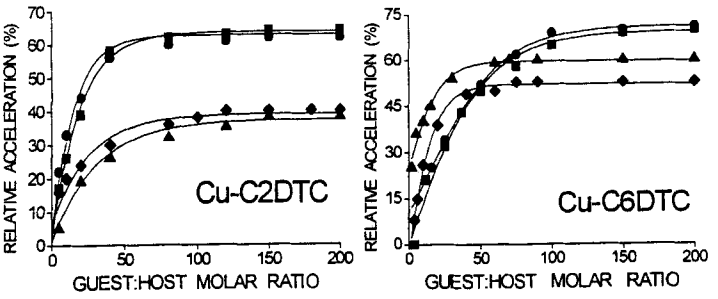


Figure 1. Relative acceleration of superoxide radical dismutation by Cu-C2DTC and Cu-C6DTC in the presence of guests **3**(■), **4**(●), **5**(▲) and **6**(◆). Solid lines are calculated curves obtained by using equation.

2 for a guest:host molar ratio of 200. This is attributed to low host-guest interaction and partial neutralization of the negative charge by hydrogen bonding with the hydroxyl groups of CD. These results demonstrate that electrostatic interactions have a significant contribution in controlling the active site accessibility of charged substrates in an enzymatic activity. Since the thermodynamic properties of the active sites for both **1** and **2** are essentially the same, variations in the catalytic activity are credited to the contribution of kinetic factors associated with the path of the substrate . Thus, the observed activity is a complex function of several factors, such as the host-guest binding constant and the positive net charge of the nitrogen atom, that determine the extent and behavior of the catalytic activity in the presence of **3-6**.

Binding Constants of 3-6 with Cu-C2DTC and Cu-C6DTC.

The saturation behavior observed (Figure 1) as the guest:host molar ratio is increased suggests that the relative acceleration is a function of the binding of the guest which fixes the anionic substrate in the vicinity of the active site. Therefore, a lower guest:host molar ratio is required to attain the maximum activity for substrates with a higher binding constant. Apparent binding constants were estimated for both hosts from the relative acceleration variations assuming 1:1 complexation stoichiometry and these results are listed in the Table.

It is clear that the host can bind the guest with two different orientations (Figure 2). In the first case, the positive charge is located on the same face of the active site, producing the maximum electrostatic attraction to the substrate (productive binding), while in the second case, the positive charge is too far from the active site (non-productive binding). Although an accurate estimate of the ratio of the two binding orientations is not possible with these experiments, it can be envisioned that the latter would be favored since this places the positively charged N atom

Table. Binding Constants (K) of Cu-C6DTC and Cu-C2DTC with Guests **3-6**.[a]

	K / M^{-1}	
GUEST	Cu-C6DTC	Cu-C2DTC
3	9.1×10^2	2.0×10^3
4	1.1×10^3	1.8×10^3
5	1.9×10^3	7.5×10^2
6	2.1×10^3	8.4×10^2

a. In *pH* 7.8 phosphate buffer at 25 °C. Relative error < 10%

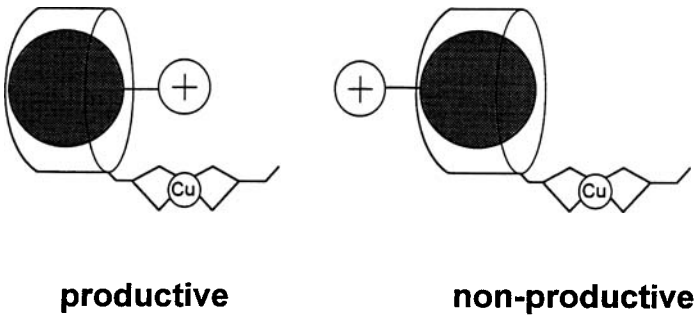


Figure 2. Productive and non-productive orientations in the inclusion complexes with Cu-CDTC.

further away from the Cu^{2+} site. However, in the total relative acceleration measured, of the contribution of the two possible structures, the productive orientation causes the highest influence. The contribution from the non-productive binding in the overall acceleration is assumably the same as that in the absence of the guest and is subtracted in the calculation of relative acceleration. Consequently, the calculated binding constants should be considered to be due to the productive binding structures alone.

Guests **5** and **6** appear to interact more strongly with Cu-C6DTC than **3** and **4**, while an opposite behavior was found for Cu-C2DTC, as shown in the Table. These results are explained by the differences in the size of the hydrophobic moiety. No direct influence of the *N*-trialkyl residues on the activity is observed, suggesting that these groups produce no steric hindrance to the approach of the substrate. On the other hand, higher K values were obtained for both triethyl containing guests (**4**, **6**) than for their trimethyl analogs (**3**, **5**) which are attributed to an enhanced non-polar interaction between the alkyl chain and the essentially hydrophobic active site.

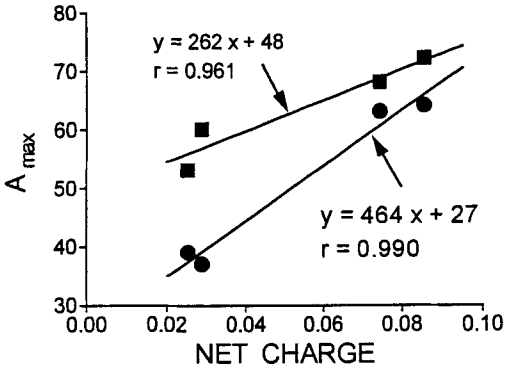


Figure 3. Influence of the net charge on the maximum relative acceleration (A_{max}) of superoxide radical dismutation by Cu-C2DTC (●) and Cu-C6DTC (■) in the presence of guests **3-6**.

Effect of the Positive Charge on the Nitrogen Atom on the Catalytic Activity .

The net charges on the nitrogen atom of **3-6** were calculated by the AM1 semiempirical method in order to understand the influence of the magnitude of the positive charge of the guests on the relative acceleration. The values obtained were **3**: 0.0854, **4**: 0.0743, **5**: 0.0288, **6**: 0.0252. Since the positive charge is fixed by the cavity, its magnitude should affect the extent of the catalytic activity under saturation conditions, that is the maximum relative acceleration observed (A_{max}) when the host exists totally as the inclusion complex. As expected, the activity increases when the charge becomes more positive, which could be explained in terms of stronger electrostatic substrate-catalyst interaction. A good correlation is found between the net charge and the maximum relative acceleration value obtained for both guests (Fig. 3).

Interestingly, A_{max} values of Cu-C2DTC are lower than that of Cu-C6DTC because the electrostatic attraction of the substrate in this regioisomer is less effective since the secondary face of CD is more open and the charge is not as close as it is in the latter host. Figure 3 also shows that A_{max} values of Cu-C2DTC are more affected by a variation in the charge as evidenced by the slope of the A_{max} *vs* net charge plot. This suggests that the effect of the change in the positive charge is more pronounced in guests with lower A_{max} and the contribution from the positive charge becomes less significant as the catalytic activity increases. This effect is expected to saturate out at a certain concentration of the net charge.

CONCLUSIONS

The total charge of the complex becomes positive as a consequence of the inclusion of a positively-charged guest into the CD cavity and facilitates a reciprocal interaction with the

substrate which is drawn towards the active site. Moreover, when the Cu(II)/Cu(I) reduction step takes place, the active site acquires a negative charge that is compensated by the included guest. This effect could aid the reoxidation step since the coordination of a second substrate is favored. The final result of this combination of kinetic effects is an enhancement in the catalytic activity.

EXPERIMENTAL

General Procedures. All chemicals were of high quality and used without further purification. NMR spectra were recorded on a Brucker AC-250 spectrometer in D_2O/DSS unless otherwise stated. Kinetic measurements were performed on a Pharmacia LKB Ultrospec III spectro-photometer, by means of the auxiliary software 'Enzyme Kinetics'.

Mono-6-methylamino-6-deoxy-ß-CD. A solution of mono-6-*O*-tosyl-ßCD[14] (1 g) in 40% aq CH_3NH_2 (20 mL) was stirred overnight, then concentrated to dryness. The crude product was redissolved in water (5 mL) and purified by cation exchange chromatography on CM Sephadex C-25 (column size: ø 1.6 X 70 cm; eluants: distiled water and 0.5 % aq. NH_3) to give the target compound in 90% yield. ^{1}H NMR (D_2O) δ 2.40 (s, 3H, CH_3), 2.75 (dd, 1H, H-6'), 2.98 (dd, 1H, H-6'), 3.40 (t, 1H, H-4'), 3.47-4.05 (m, 39H, H-2, H-3, H-4, H-5, H-5', H-6), 5.05 (m, 7H, H-1); ^{13}C NMR (D_2O) δ 36.2 (CH_3), 52.8 (C-6'), 61.5 (C-6), 71.5 (C-5'), 72.9, 73.21 (C-3, C-5), 74.2 (C-2), 82.4 (C-4), 85.2 (C-4'), 103.0 (C-1); FABMS *m/z* 1169.3 $(M+Na)^+$.

Triethylammonium Mono-6-methylamino-6-deoxy-ß-CD-dithiocarbamate (2). CS_2 (100 μL) was added to a solution of mono-6-methylamino-ßCD (500 mg) in 2% aq NEt_3 (5 mL) and the resulting emulsion was stirred for 3 h at rt, then concentrated to dryness. The crude product was dissolved in water (5 mL) and precipitated by addition of acetone (250 mL). The resulting white solid was collected by filtration, redissolved in water (5 mL) and precipitated with acetone to give **2** (520 mg, 89 %): mp 198-200 °C (dec.); UV (H_2O) λ_{max} 255 ($\epsilon = 2.3X10^4$) , 290 ($\epsilon = 3.0X10^4$); ^{1}H NMR (D O/DSS) δ 3.14 (s, 3H, NCH_3), 3.39-3.93 (m, 44H, H-2, H-3, H-4, H-4', H-5, H-6, H-6'), 4.91-5.00 (m, 7H, H-1); ^{13}C NMR (DMSO-d_6/TMS) δ 33.1 (NCH_3), 52.4 (C-6'), 62.9 (C-6), 74.5, 74.7, 76.0 (C-2, C-5, C-3), 83.2, 83.8 (C-4, C-4'), 103.9, 104.7 (C-1, C-1'), 207.4 (CSS). FABMS *m/z* 1267.1 $(M-NHEt_3+2Na)^+$, 1324.3 $(M+H)^+$.

General Method for the Synthesis of the Guests. The guests were prepared by exhaustive alkylation of the corresponding amines (cyclohexylamine and *p*-toluidine) with

a 10 fold molar excess of the alkyl iodide in benzene under reflux conditions. Perethylation of *p*-toluidine was carried out in the presence of an alkaline catalyst (50% aq KOH). The products were recrystallized twice from acetone and dried under vacuum to give **3-6** in 50-70 % overall yield. ^{1}H NMR (D_2O/DSS) δ **3**: 1.1-1.5 (m, 5H, H-2, H-3, H-4), 1.65 (m, 1H, H-4'), 1.79 (m, 2H, H-3'), 2.02 (m, 2H, H-2'), 3.03 (s, 9H, NCH_3),3.13 (tt, 1H, H-1); **4**: 1.27 (t, 9H, CH_3), 1.3-1.5 (m, 5H, H-2, H-3, H-4), 1.67 (m, 1H, H-4'), 1.82 (m, 2H, H-3'), 2.06 (m, 2H, H-2'), 3.11 (m, 1H, H-1), 3.13 (q, 6H, NCH_2); **5**: 2.41 (s, 3H, p-CH_3), 3.65 (s, 9H, NCH_3), 7.47 (dt, 2H, H-3), 7.73 (dt, 2H, H-2); **6**: 1.55 (t, 9H, CH_3), 2.78 (s, 3H, p-CH_3), 4.29 (q, 6H, NCH_2), 7.80 (dt, 2H, H-3), 8.15 (dt, 2H, H-2). ^{13}C NMR (D_2O/DSS) δ **3**: 26.72 (C-3), 27.18 (C-4), 30.78 (CH_3), 33.23 (C-2) 53.28 (C-1); **4**: 13.59 (CH_3), 26.60 (C-3), 27.24 (C-4), 31.65 (C-2), 42.40 (CH_2), 59.26 (C-1); **5**: 22.78 (p-CH_3), 54.81 (NCH_3), 122.14 (C-2), 133.56 (C-3), 143.97 (C-4), 154.02 (C-1); **6**: 9.36 (CH_3), 21.86 (p-CH_3), 59.79 (NCH_2), 121.98 (C-2), 132.11 (C-3), 142.65 (C-4), 155.27 (C-1).

SOD-like Activity. The SOD-like activity was determined under the same conditions as those reported elsewhere.[5] Superoxide radical was generated by the xanthine-xanthine oxidase system; phosphate buffer pH 7.8 (10 mM), nitroblue tetrazolium chloride (NBT) (2.5 μM) and xanthine (10 μM) was used as the reaction mixture. NBT reduction by $O_2^{\cdot-}$ was spectrophotometrically monitored at 560 nm. The copper (II) complexes were prepared *in situ* in 20% aq DMSO and their concentrations were fixed at their previously measured IC_{50} value (4.6 μM for Cu-C2DTC[5] and 60 μM for Cu-C6DTC), while the guest concentration was 2-200 fold greater than these values. The relative acceleration of $O_2^{\cdot-}$ dismutation was referred to the activity of the complex alone and was the average of 5 determinations with relative errors less than 10 %.

Binding Constants. Binding constants (*K*) were determined considering the formation of a 1:1 inclusion complex. The complexation equilibrium under study is described by the following equation:

$$CD + G \quad \approx \quad CD\text{-}G$$

where CD, G and CD-G represent the catalyst, the guest and the inclusion complex respectively. The binding constants were defined by equation 1:

$$K = \frac{I}{(C_0 - I)(G_0 - I)}$$

where C_0 and G_0 represent the initial concentrations of host and guest respectively and I represents the inclusion complex concentration in the equilibrium. Letting A be the catalytic

activity of the complex for several guest:host molar ratios and A_{max} be the maximum observed activity, the inclusion complex concentration was expressed as a function of the activity by the equation: $I = C_0 A / A_{max}$. Substituting $G_0 = R\ C_0$ in equation 1, where R is the guest:host molar ratio and rearranging gives:

$$K = \frac{A / A_{max}}{C_0(1 - A / A_{max})(R - A / A_{max})}$$

Therefore,

$$A = A_{max} \frac{C_0(R+1) + \frac{1}{K} - \sqrt{(C_0(R+1) + \frac{1}{K})^2 - 4RC_0^2}}{2C_0}$$

Equation 3 describes the theoretical behavior of the catalytic activity as a function of guest:host molar ratio. K values were calculated by least-squared fitting of eq 3 to the experimental points.

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