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Complexation of Bis(morpholyldithiocarbamato)copper(II), a Superoxide Scavenger, in β -Cyclodextrins

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Bis(morpholyldithiocarbamato)copper(II), Cu(MorDTC)₂, has been studied as a scavenger of the superoxide anion, a reactive radical that can cause very negative effects *in vivo*. The scavenging takes place through a superoxide dismutase (SOD)-like process. The copper(II) compound has a poor solubility in water. The interaction of Cu(MorDTC)₂ with β -cyclodextrin (β -CD) and its dimethyl derivative (dm β -CD) was studied, resulting in an increase by one order in water solubility and the SOD-like activity of Cu(MorDTC)₂. The inclusion constants were determined spectrophotometrically at 435 nm and 300 K. For β -CD, the values of $\log K_{11} = 3.96 (\pm 0.01)$ and $\log K_{21} = 7.47 (\pm 0.04)$ were obtained. With dm β -CD, $\log K_{11} = 3.73 (\pm 0.08)$ and $\log K_{21} = 6.30 (\pm 0.01)$ were the values achieved.

Keywords: Copper(II); Dithiocarbamate; Cyclodextrin; Inclusion complex; SOD-like activity

INTRODUCTION

Cyclodextrins (CDs) are a class of cyclic oligosaccharides composed of $\alpha(1 \rightarrow 4)$ -linked D-glucopyranose units in the ⁴C₁ chair conformation. The overall form of the molecules is a truncated cone with an essentially hydrophobic cavity. Such a structure allows them to form stable inclusion complexes with a wide variety of guests [1].

CDs have been widely used in pharmaceutical formulations of drugs to increase their water solubility (e.g. itraconazole) and stability (e.g. PGE₁), to mask odor (garlic oil) and flavor, to perform a drug release control (loteprednol) and to favor their absorption (e.g. piroxicam) [2]. In most cases such formulations are based on unmodified CDs mixed with the corresponding drug.

When a high increase in water solubility is desired dimethyl- or hydroxypropyl- β -CDs are used [2].

At our laboratory bis(morpholyldithiocarbamato)copper(II), Cu(MorDTC)₂, has been studied as a scavenger of superoxide anion [3], a reactive radical that can cause very negative effects *in vivo*. The radical is scavenged based on a process similar to that carried out *in vivo* by superoxide dismutase (Cu,Zn-SOD). This metalloenzyme is extremely efficient and dismutates superoxide radical into O₂ and H₂O₂ [4].

Cu(MorDTC)₂ is a neutral copper(II) complex that is poorly soluble in water, with a K_{sp} less than 10⁻¹⁵, which causes problems for the experimental studies in aqueous solutions. The goal of the present paper is to overcome the extremely low solubility of Cu(MorDTC)₂ in water by its inclusion in the appropriate cyclodextrin without affecting its ability to dismutate superoxide radical in an SOD-like fashion.

RESULTS AND DISCUSSION

PM3 semiempirical calculations were performed in order to analyze the possible inclusion of Cu(MorDTC)₂ in cyclodextrin cavities.

In order to avoid the nonparametrization of the copper(II) compound by this method, the calculations were carried out for the corresponding isostructural zinc(II) compound. The similarity of the ionic radii of Cu(II) and Zn(II) was also considered in making this substitution.

Initially, the size of the zinc(II) compound was calculated. This planar compound, with a quasi-rectangular form is 4.75 Å wide and 14.01 Å long, as depicted in Fig. 1. According to these parameters,

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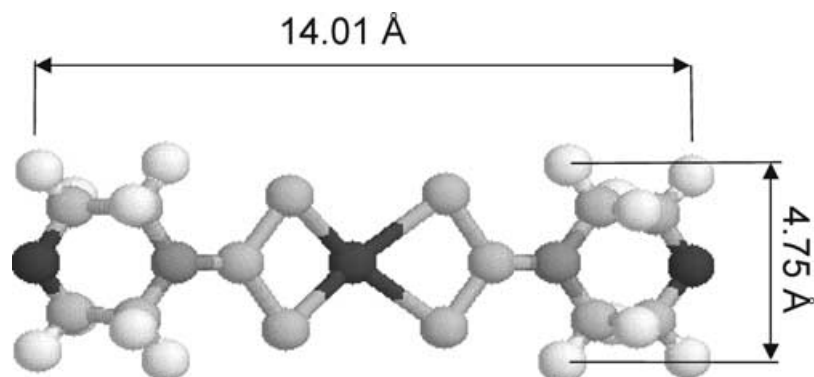


FIGURE 1 Main distances in $\text{Zn}(\text{MorDTC})_2$ obtained by semiempirical PM3 calculations.

$\text{Cu}(\text{MorDTC})_2$ should not be expected to form a stable inclusion complex with γ -CD since its width is too small and its length too long; however, its width is very similar to that of the diameter of the α -CD cavity. Evidently, inclusion should not be expected either in the α -CD cavity due to the very high similarities in sizes between host and guest. Therefore, the best inclusion is to be expected in β -CD. Such results were obtained when the inclusion of $\text{Zn}(\text{MorDTC})_2$ in α -, β - and γ -CD was studied using semiempirical calculations.

In order to perform the calculation of the inclusion process, the following steps were considered:

1. Hydration of both host and guest (Fig. 2a),
2. Disruption of the hydration forms of both host and guest when they approach each other (Fig. 2b),
3. Formation of a 1:1 inclusion complex (Fig. 2c).

The potential energy of the 1:1 inclusion complex is -87 kJ mol^{-1} . This value indicates the formation of a fairly stable 1:1 inclusion complex. Once the compound is included the β -CD cavity is deformed. Along the plane of the guest the diameter of the cavity increases up to 8.21 \AA , with a decrease down to 5.56 \AA along the perpendicular section. This way the β -CD cavity adopts an oval form.

According to the calculations the inclusion of the guest, $\text{Zn}(\text{MorDTC})_2$, completely inside the β -CD, encroaching on the primary rim, results in an unstable state of the complex. Therefore, it should be assumed that a 2:1 inclusion complex can be formed, with both secondary rims facing the metal center. We could not calculate the formation of a 2:1 inclusion complex using Mopac software since this latter complex contains more than 125 atoms, the maximum allowed for this software. Nevertheless, the 2:1 inclusion complex was designed (Fig. 3) and the distance between the secondary rims of both β -CDs was estimated to be approximately 5.4 \AA .

Once the formation of a 2:1 β -CD: $\text{Zn}(\text{MorDTC})_2$ inclusion complex had been predicted, we decided to confirm it experimentally with $\text{Cu}(\text{MorDTC})_2$. The preliminary criterion, of practical importance, was to observe any possible increase in the water solubility of $\text{Cu}(\text{MorDTC})_2$ in the presence of the cyclodextrins. This compound can only be dissolved in water when formed *in situ* by slow dropwise addition of the ligand over the Cu(II) salt and with constant stirring. Under such strict conditions a $6.0 \times 10^{-5} \text{ M}$ solution can be attained, which is only stable for very few minutes.

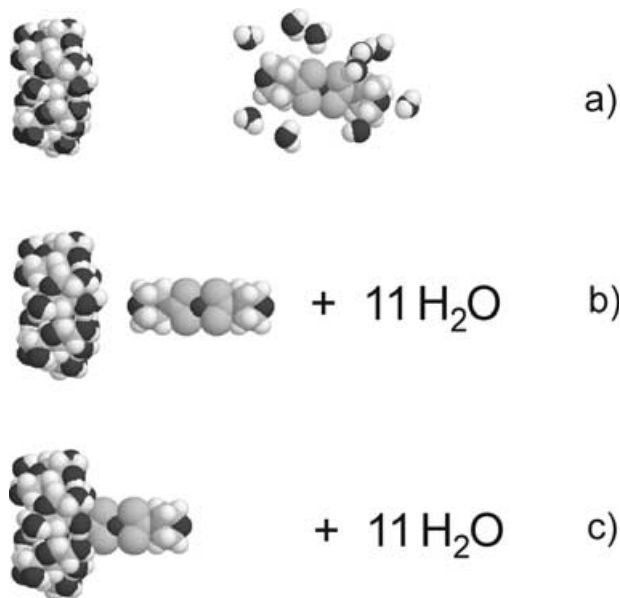


FIGURE 2 Results of the calculation of the inclusion process, considering the following steps: (a) hydration of both host and guest; (b) disruption of the hydration forms of both host and guest when they approach each other and (c) formation of a 1:1 inclusion complex.

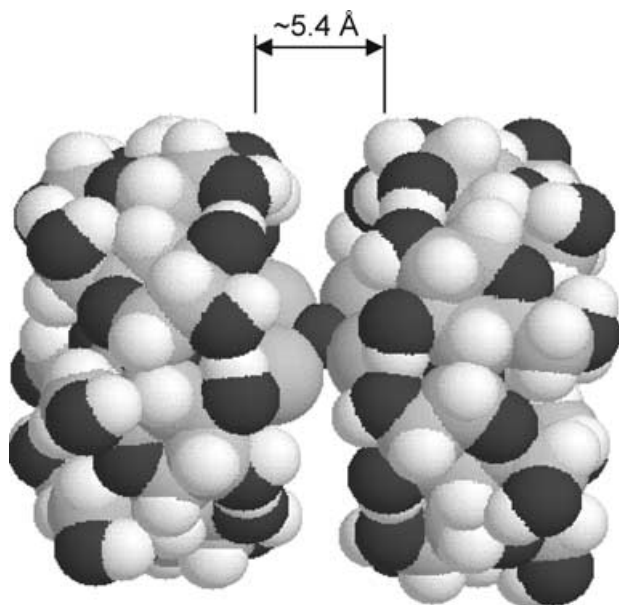
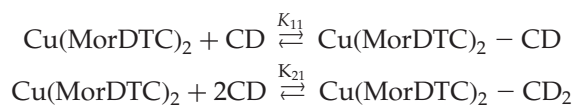


FIGURE 3 Design of the 2:1 inclusion complex with the estimated distance between the secondary rims of both β -CDs.

The copper(II) compound was formed *in situ* in 10^{-2} M solutions of α -, β - and γ -CD and dm β -CD (per-2,6-di-O-methyl- β -CD). As expected, no increase in water solubility was observed either in α -CD or in γ -CD. With β -CD and dm β -CD the highest concentration of Cu(MorDTC)₂ attained was 1.07×10^{-4} M and 2.02×10^{-4} M, respectively. The resulting solutions were stable for several hours, with no variations in the absorbance of the solutions at 435 nm.

The inclusion caused variations in the intensity of the 435 nm band with no shift in the maximum, but with different trends depending on the host. Cu(MorDTC)₂ in water gives a molar absorptivity of $12400 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 435 nm. When β -CD was used as the host an increase in the intensity ($\epsilon = 14000 \text{ L mol}^{-1} \text{ cm}^{-1}$) was observed, in contrast to the decrease attained with dm β -CD ($\epsilon = 11500 \text{ L mol}^{-1} \text{ cm}^{-1}$).

The inclusion constants of Cu(MorDTC)₂ in β -CD and dm β -CD were determined spectrophotometrically at 300 K and 435 nm using LETAGROP software [5]. Since the formation of a 2:1 host:guest complex was predicted, the following equilibria were considered:



The experimental absorbance *vs.* concentration plot was fitted with this model. For β -CD the values of $\log K_{11} = 3.96 (\pm 0.01)$ and $\log K_{21} = 7.47 (\pm 0.04)$ were obtained. With dm β -CD values of $\log K_{11} = 3.73 (\pm 0.08)$ and $\log K_{21} = 6.30 (\pm 0.01)$ were achieved.

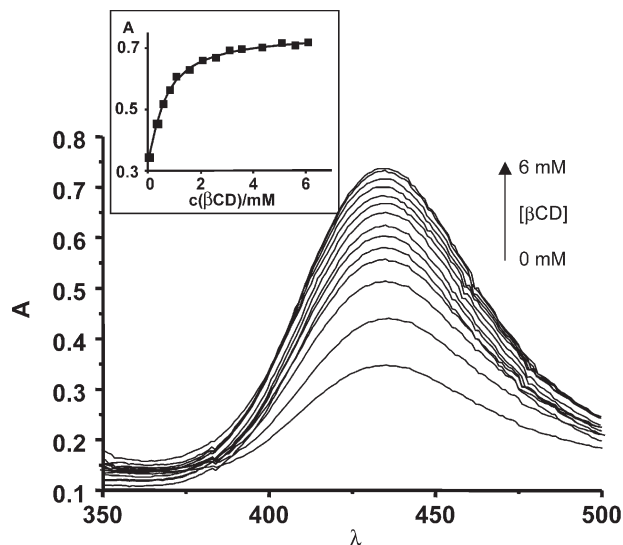


FIGURE 4 Increase of absorbance in the spectra of the Cu(MorDTC)₂- β -CD system for $50 \mu\text{M}$ solutions of Cu(MorDTC)₂ and concentrations of β -CD within the range of 0 and 6 mM. A binding plot of the variation of absorbance *vs.* [CD] is included.

Fig. 4 depicts the variations in the spectra of Cu(MorDTC)₂ in the presence of increasing concentrations of β -CD and the resulting binding plot. The slightly lower stability achieved with dm β -CD could be due to the steric hindrance caused by the methyl groups of the guest.

The obtained inclusion constants indicate the formation of a 2:1 complex stable enough to be used for the solubilization of Cu(MorDTC)₂, as has been already demonstrated. According to the results obtained by semiempirical calculations the copper(II) center should not be affected by the inclusion and, therefore, is able to interact with the superoxide radical. In order to confirm this prediction the complex included in β -CD and dm β -CD was studied for any SOD-like activity.

Superoxide radical was generated enzymatically with the xanthine/xanthine oxidase system and the interaction was followed spectrophotometrically at 560 nm, using nitroblue tetrazolium chloride as indicator [6]. The activity is expressed as IC₅₀, the concentration (μM) of the complex necessary to dismutate 50% of the evolved superoxide radical. The values obtained in this work are listed in Table I,

TABLE I IC₅₀ values for the studied systems

System	IC ₅₀ (μM)	References
Cu(MorDTC) ₂ - β -CD ₂	0.5	This work
Cu(MorDTC) ₂ -dm β -CD ₂	0.9	This work
Cu(MorDTC) ₂	3.2	Ref. [3]
Cu(β C2DTC) ₂	4.6	Ref. [7]
Cu(β C6DTC) ₂	60	Ref. [9]

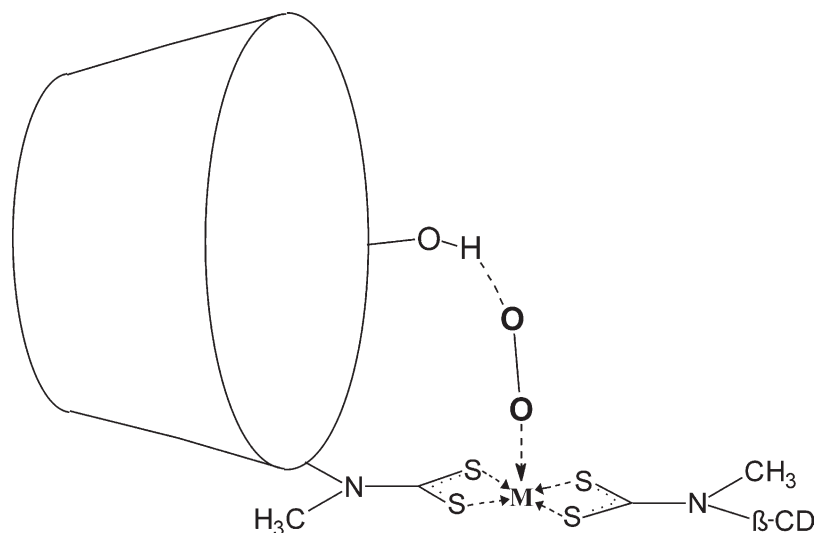


FIGURE 5 Schematic representation of H-bond formation between the OH groups of the secondary rim and the superoxide radical as the substrate reported for a copper(II) complex of β -CD monosubstituted with a dithiocarbamate group [9].

together with those of some other systems used for comparison purposes.

The observed increase in the SOD-like activity (lower IC_{50} value) of the included copper(II) complex requires an explanation. According to our previous studies [3,7–12] copper(II) mimetics of Cu,Zn-SOD should observe, among others, the following characteristics:

1. The copper(II) atom should be able to reversibly reduce to copper(I).
2. The ligand should have groups capable of forming hydrogen bonds with the substrate.

The cyclic voltammogram of this copper(II) compound in DMSO gave a quasi-reversible oxidation peak at 0.44 V *vs.* Ag/AgCl [8]. $Cu(MorDTC)_2$ possesses no group able to form hydrogen bonds with the substrate which, according to the above statements, is an important condition to be considered for a Cu,Zn-SOD mimic. But when it is included in β -CD the OH groups of the host should be able to fulfil the second condition.

In previous studies β -CDs monosubstituted with a dithiocarbamate group on the primary or the secondary rims were synthesized and their copper(II) compounds were studied [7,9]. When the dithiocarbamate group was substituted on the secondary rim, $Cu(\beta\text{-C2DTC})_2$, the observed SOD-like activity of the copper(II) compound was one order higher ($IC_{50} = 4.6 \mu\text{M}$) than on the primary rim, $Cu(\beta\text{C6DTC})_2$, ($IC_{50} = 60 \mu\text{M}$). This difference was attributed to the higher acidity of the OH groups on the secondary rim of β -CD and, therefore, its possibility to form stronger H-bonds with the superoxide radical as depicted in Fig. 5.

H-bond formation is more sterically limited in the case of β -CD monosubstituted with a dithiocarbamate group ($Cu(\beta\text{C2DTC})_2$ and $Cu(\beta\text{C6DTC})_2$) where the OH groups of the CD moieties lie asymmetrically with respect to Cu(II) (Fig. 5). This should be the reason why the SOD-like activity is higher for the $Cu(MorDTC)_2\text{-}\beta\text{-CD}_2$ complex, in which Cu(II) is symmetrically surrounded by the secondary OH groups of both β -CD, than for the compound depicted in Fig. 5. On the other hand, the fact that the SOD-like activity of $Cu(MorDTC)_2\text{-}\beta\text{-CD}_2$ is also higher than when the copper(II) compound is not complexed ($Cu(MorDTC)_2$) indicates that it is precisely the secondary rim which is pointing toward the copper(II) center (Fig. 6), confirming the prediction made by the semiempirical calculations. This is due to the fact that secondary OH groups are more acidic and can, therefore, form stronger H-bonds with the substrate, as reported earlier for the copper(II) complexes of β -CDs monosubstituted with a dithiocarbamate group, $Cu(\beta\text{C2DTC})_2$ and $Cu(\beta\text{C6DTC})_2$, [7,9].

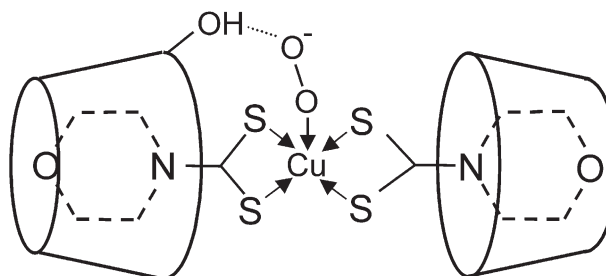


FIGURE 6 Schematic representation of $Cu(MorDTC)_2$ included in β -CD, where the OH groups of the secondary rim could form H-bonds with the substrate.

Naturally, the inclusion complex with $\text{dm}\beta\text{-CD}$, $\text{Cu}(\text{MorDTC})_2\text{-dm}\beta\text{-CD}_2$, exhibits a SOD-like activity lower than $\text{Cu}(\text{MorDTC})_2\text{-}\beta\text{-CD}_2$ due to the restrictions in H-bond formation introduced by the methyl groups.

EXPERIMENTAL

The cyclodextrins and the other chemicals, all of high quality, were purchased from Aldrich and used without further purification. Morpholyldithiocarbamate, sodium salt, NaMorDTC , was synthesized as reported elsewhere [3]. CuSO_4 aqueous solutions (5 mM) were used for the *in situ* formation of $\text{Cu}(\text{MorDTC})_2$. The spectrophotometric determinations were performed on a Ultrospec III (Pharmacia-LKB) equipment.

The water solubility of $\text{Cu}(\text{MorDTC})_2$ in the presence of the different cyclodextrins was followed spectrophotometrically at 435 nm. The cyclodextrin (10 mM) was initially dissolved in the presence of phosphate buffer pH 7.4, and then 10 mM MorDTC and 5 mM CuSO_4 aqueous solutions were added (the latter dropwise and with constant stirring) in order to obtain 50–300 μM solutions of $\text{Cu}(\text{MorDTC})_2$. For the spectrophotometric determination of the inclusion constants of $\text{Cu}(\text{MorDTC})_2$ in $\beta\text{-CD}$ and $\text{dm}\beta\text{-CD}$, 50 μM solutions of $\text{Cu}(\text{MorDTC})_2$ in the presence of the cyclodextrin were prepared, varying the concentration of the latter within the range of 0 and 6.5 mM for the system (70 mM for $\text{dm}\beta\text{-CD}$). For the highest concentration of the guest no further variations in the absorbance of the system was observed. The inclusion constants of $\text{Cu}(\text{MorDTC})_2$ in $\beta\text{-CD}$ and $\text{dm}\beta\text{-CD}$ were determined at 300 K and $\lambda = 435\text{ nm}$ using LETAGROP software [5]. This program uses the concentrations and molar absorptivity values of both host and inclusion complex. With these data the program calculates the absorbance of the solutions, fits the variations of absorbance of the system by the least squares method and calculates the corresponding inclusion constants.

The SOD-like activity was determined according to the method described by Fridovich [6] which consists of generating enzymatically the superoxide radical with the xanthine/xanthine oxidase system and following the interaction spectrophotometrically at 560 nm, using nitroblue tetrazolium chloride (NBT) as indicator. Phosphate buffer pH 7.8 solutions (10 mM) containing xanthine (10 μM) and

NBT (2.5 μM) were prepared and the amount of xanthine oxidase necessary to achieve slopes of $\sim 0.025 \Delta\text{Abs}$ per min was added. EDTA was not used in order to avoid chelating competition with the dithiocarbamate. The kinetic measurements were performed on the spectrophotometer using its Enzyme Kinetics (EK) software. The assay time was fixed at no less than 10 min, with measurements each minute. The obtained ΔAbs per min slopes had, in all cases, a linearity greater than 0.993. Each series of experiments was carried out with four cuvettes, synchronized in time by the EK program; the first cuvette was always the xanthine oxidase system without the assay. The % of inhibition was calculated related to the slope of this first cuvette (ref. slope) using Eq. (1):

$$\% \text{ inhibition} = (\text{assayslope} - \text{ref.slope}) \times 100 / \text{ref.slope} \quad (1)$$

The IC_{50} values were determined by regression analysis and interpolation of the % inhibition *vs.* assay concentration curve, for no fewer than five experimental points for each system, with inhibition values within the range of 20 to 80%. Inhibitions out of this range tend to have a nonlinear behavior. In all cases, a linearity greater than 0.98 was achieved. The PM3 (MNDO-PM3 Hamiltonian) semiempirical calculations were performed on a Pentium 3 PC using Mopac (Version 6.0) software using a convergence factor of 100. The geometries were previously optimized on Hyperchem 7.0 program.

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