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Characterization and superoxide dismutase activity of copper(II) complexes of 6A,6X-difunctionalized β-cyclodextrins

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Thermodynamic and EPR spectroscopic measurements were carried out in aqueous solution on copper(II) complexes of β-cyclodextrins 6A,6X-difunctionalized (X = B, C or D) with histamine (imidazole-4-ethanamine). The O_2^- scavenger activity of these systems was determined through both enzymatic and photohaemolysis assays. An attempt was made to correlate the scavenger ability with the geometrical features of the copper(II) complexes. The determination of the stability constants of the copper(II) complexes with 6A,6B-bis[2-(imidazol-4-yl)ethylamino]-6A,6B-dideoxy-β-cyclodextrin revealed the speciation under the experimental conditions.

Cyclodextrins (cds) have been widely used as enzyme models. The possibility of mimicking a larger number of enzymes has been provided by the appropriate functionalization of cds by enhancing their enzyme-like activity.[1-3] Furthermore, the functionalization with moieties able to co-ordinate a metal ion has provided models for metalloenzymes.[4,5]

In this context a number of inert and substitution-labile metal complexes of functionalized cds have been described.[4-7] The presence of the cavity may modify some properties of the metal-ion complex. An interesting case is the Fe_4S_4 clusters [8] obtained using a functionalized cd. The stability of this cluster in water is greater than other synthetic clusters without a cd cavity which, instead, are quickly hydrolysed. In the cd–Fe_4S_4 cluster the OH^- can probably compete with the H_2O molecules thus protecting the cluster against hydrolysis.[8]

More recently, our interest has been attracted by the possible use of copper(II) complexes of β-cyclodextrin derivatives as SOD-mimicking compounds (SOD = superoxide dismutase).[9,10] In fact, copper(II) complexes of monofunctionalized cyclodextrins show a protective action against erythrocyte photohaemolysis induced by oxygen radicals photochemically generated by a non-steroidal antiinflammatory drug, ketoprofen.[11] This activity has been attributed to the scavenger ability of copper(II) complexes of these substituted β-cyclodextrins towards oxygen radicals.[9]

Effectively to mimic superoxide dismutase, a transition-metal complex should have the following properties: (*a*) solubility in water; (*b*) stability; (*c*) flexible co-ordination geometry; (*d*) activity between pH 6 and 8; (*e*) 'open' co-ordination with rapidly exchanging water molecules; (*f*) redox centre with appropriate redox potential as catalyst for superoxide dismutation ($2O_2^- + 2H^+ \rightleftharpoons O_2 + H_2O_2$). Bearing this in mind, the three regioisomers 6A,6X-bis[2-(imidazol-4-yl)ethylamino)]-6A,6X-dideoxy-β-cyclodextrin (L^{AX}, X = B, C or D) have been previously synthesized and their conformational features determined.[12] The use of these regioisomers of difunctionalized cds is a simple way to tune the metal co-ordination.

Here we report the O_2^- scavenger activity of functionalized cyclodextrin copper(II) complexes determined by means of the classical indirect assay.[10] Furthermore the protective action of these complexes towards photosensitization processes in the presence of a membrane such as red blood cells was determined. The stability constants of the metal complexes were determined in aqueous solution by means of potentiometric titration in order to characterize the species, thus permitting us to deal with the complexes which really exist under the experimental conditions of the enzymatic assay and in photohaemolysis experiments. Spectroscopic (EPR and UV/VIS) measurements were also carried out in order to find a correlation between the catalytic activity and the geometrical features of the complexes.

Experimental

Materials

Copper(II) nitrate was a Merck product 'reinst'. The concentration of stock solutions of this salt was determined by ethylenedinitrilotetraacetate titrations with murexide as an indicator. All solutions were prepared with CO_2-free freshly distilled (four times) water. The ionic strength was adjusted to 0.10 mol dm^{-3} by adding KNO_3 (Suprapur Merck). Grade A glassware was employed throughout. Ketoprofen, 2-[3-benzoylphenyl]propionic acid, xanthine, xanthine oxidase, cytochrome c and bovine superoxide dismutase were obtained from Sigma. All other chemicals were reagent grade. Phosphate-buffered saline solution (pH 7.4) consisted of a 10 mmol dm^{-3} phosphate buffer and 0.135 mol dm^{-3} NaCl solution. The compounds L^{AX} (X = B, C or D) were synthesized as previously reported.[12]

Electromotive force measurements

The potentiometric measurements were carried out by means of two fully automated computer-controlled meters (Metrohm E 654) using a combined glass microelectrode (Metrohm 125). All experiments were carried out at 25.0 + 0.2 °C using 2.5 cm^3 thermostatted cells. All solutions were magnetically stirred and maintained under an atmosphere of inert nitrogen. The other experimental details were as previously reported.[13,14]

Calculations

The calculations concerning the calibration of the electrode system were performed using the computer program ACBA.[15] The program SUPERQUAD[16] was used to handle all other data. The distribution diagram of the complex species *versus* the pH was obtained by means of the computer program DISDI.[17]

Spectroscopic measurements

The EPR spectra of frozen solutions were obtained by a Bruker ER 200D instrument driven by the ESP 3220 data system. All spectra were recorded at 150 K achieved with the use of a standard low-temperature apparatus. Solutions of 5 mol dm^{-3} copper(II) complexes were prepared by mixing aqueous solutions of $^{63}Cu(NO_3)_2 \cdot 6H_2O$ and the pertinent proligand in 1:1 molar ratio and adjusting to pH 7 by adding KOH. Up to 10% methanol was added to increase resolution which is known to be poor for aqueous solutions. The diphenylpicrylhydrazyl (dpph) radical ($g = 2.0036$) was used to standardize the klystron frequency, the magnetic field being monitored by a Bruker ER 035M gauss meter. Spin-Hamiltonian parameters were obtained by carefully simulating the experimental spectra making use of a computer program principally devised by Pilbrow and Winfield.[18] Angular overlap model (AOM) parametrization was used to compute electronic transitions as well as principal g and A values as a function of the copper–nitrogen bond angles, making use of a computer program devised by Bencini and Gatteschi.[19] From the comparison between calculated and experimental values, information on the distortion undergone by the co-ordination polyhedra of the copper(II) complexes was inferred. Electronic spectra at room temperature were recorded on a Hewlett-Packard HP 8452 spectrophotometer in 1.0 cm optical path quartz cells by using the aqueous solutions from the EPR study.

Superoxide dismutase assay

The SOD-like activity was determined by the indirect method of cytochrome c reduction.[20] Solutions containing $Cu(NO_3)_2$ (10^{-5}–10^{-7} mol dm^{-3}) and the proligand (L) in phosphate buffer (5×10^{-3} mol dm^{-3}, pH 7.4) were used. The proligand was used in a sufficient L:M ratio (5:1) to achieve complete complex formation. Superoxide anion was enzymatically generated by the xanthine–xanthine oxidase system and spectrophotometrically detected by monitoring the formation of reduced cytochrome c which absorbs at 550 nm.

The reaction mixture comprised cytochrome c (80 μmol dm^{-3}) and xanthine (50 μmol dm^{-3}) in phosphate buffer (10 mmol dm^{-3}) at pH 7.4. An appropriate amount of xanthine oxidase was added to the reaction mixture (2 cm^3) to cause a change in absorbance at 550 nm, ΔA_{550} of 0.024 min^{-1}. This corresponded to a O_2^- production rate of 1.1 μmol dm^{-3} min^{-1}. The cytochrome reduction rate was measured in the presence and absence of the investigated complexes for 300 s. All measurements were carried out at 25 ± 0.2 °C using 1 × 1 cm thermostatted cuvettes, in which solutions were magnetically stirred. In separate experiments urate production by xanthine oxidase was spectrophotometrically monitored at 298 nm, ruling out any inhibition of xanthine oxidase activity. The I_{50} (the concentration which causes 50% inhibition of cytochrome c reduction) of copper(II) complexes at pH 7.4 was calculated.

Irradiation conditions and photohaemolysis experiments

Irradiation was performed in the 310–390 nm range. The fluence at the irradiation position was about 800 μW cm^{-2} and the photon flux incident on a 3 cm^3 solution in the quartz cuvettes (10 mm optical path) was 5×10^{15} quanta s^{-1}. The light intensity was measured by means of an iron(III) oxalate actinometer[21] and by a Spectroline model DRC-100X digital radiometer equipped with a DIX-365 sensor, spectral range 320–380 nm. All experiments were carried out at 25 ± 0.2 °C in phosphate-buffered saline (20 mmol dm^{-3}), at pH 7.4. The experimental procedures of irradiation and the light-intensity measurements have been described previously.[22,23] Red blood cells were prepared by washing, in phosphate-buffered saline, samples of out-of-date (not more than 15 d from the date stated) packed human erythrocytes, supplied by the local blood bank. Each series of tests was performed with aliquots from the same sample of blood. Photohaemolysis experiments were carried out by measuring the decrease in absorbance at 650 nm, since the optical density is directly proportional to the number of intact red blood cells.[11] The haemolysis data refer to the times measured starting from the beginning of the irradiation (delayed haemolysis time); the temperature was maintained at 25 ± 0.2 °C. The irradiated red blood cell suspensions in phosphate-buffered saline contained ketoprofen (4×10^{-5} mol dm^{-3}), copper(II) nitrate (10^{-6}–10^{-7} mol dm^{-3}) and proligand in a variable ratio with respect to copper(II) ion; the metal ion and the proligand were premixed. Irradiation times ranged between 5 and 10 min and the delayed semihaemolysis times (t_{50}) were in the range 45–600 min. No lysis was observed during this time when cells were irradiated in the absence of ketoprofen and/or metal complexes or incubated with these compounds in the dark. From the ratio between the t_{50} values in the presence and in the absence of copper(II) complexes a protection factor (F) was calculated. No variation in F was observed by varying the irradiation time. Other experimental details have been previously reported.[9,11]

Results and Discussion

The thermodynamic investigation was carried out on the 6A,6B-bis[2-(imidazol-4-yl)ethylamino]-6A,6B-dideoxy-β-cyclodextrin (L^{AB}) only, because of the low yield of the synthetic route for this class of compounds.[12] The stability constants were determined to correlate the activity observed in the biological assay to the species existing under the experimental conditions of these assays.

The protonation constants of L^{AB} together with those of the 6-[2-(imidazol-4-yl)ethylamino]-6-deoxy-β-cyclodextrin (L) and histamine (imidazole-4-ethanamine, hist) are reported in Table 1. The comparison of the protonation constants of L^{AB} with those of hist and L previously investigated[24] can help us to understand the lower stability of the cyclodextrin copper(II) complex. The first protonation step of L^{AB} (at the amine nitrogen) shows a log K value lower than that of histamine. A lower value is also found for L.[24] On the basis of NMR measurements at different pH values as well as of enthalpy and entropy values, we have previously concluded that this difference is due to the presence of an intrachain hydrogen bond in the species L. Thus, protonation is thermodynamically unfavourable because it involves breaking of this hydrogen bond. The same explanation can be suggested for L^{AB} and the NMR investigation confirms this conclusion.[12] The second protonation constant, which involves the amino group of the second residue, is lower than that of histamine. This difference can be attributed to the same effect. Moreover, the statistical factor and conformational changes which modify the possible stacking interaction between the two imidazole rings, present in the unprotonated species, may explain the lower protonation constant of the second step. The other two protonation steps, involving imidazole nitrogens, are not significantly different from those of L.

Table 1 Protonation constants of L^{AB} at 25 °C and 0.1 mol dm^{-3} (KNO_3)

Equilibrium	log K	Ref.
$hist + H^+ \rightleftharpoons [Hhist]^+$	9.80	24
$[Hhist]^+ + H^+ \rightleftharpoons [H_2hist]^{2+}$	6.08	24
$L + H^+ \rightleftharpoons [HL]^+$	8.01	24
$[HL]^+ + H^+ \rightleftharpoons [H_2L]^{2+}$	5.81	24
$L^{AB} + H^+ \rightleftharpoons [HL^{AB}]^+$	8.13(1)	This work
$[HL^{AB}]^+ + H^+ \rightleftharpoons [H_2L^{AB}]^{2+}$	7.24(1)	This work
$[H_2L^{AB}]^{2+} + H^+ \rightleftharpoons [H_3L^{AB}]^{3+}$	5.86(1)	This work
$[H_3L^{AB}]^{3+} + H^+ \rightleftharpoons [H_4L^{AB}]^{4+}$	5.17(1)	This work

Table 2 shows the stability constants of the copper(II)–L^{AB} complexes. The main species is $[CuL^{AB}]^{2+}$ starting from pH 6 (see Fig. 1) and is less stable than $[Cu(hist)_2]^{2+}$. Also in this case the breaking of the intrachain hydrogen bond can be invoked to explain the lower stability constant of $[CuL^{AB}]^{2+}$ with respect to that of $[Cu(hist)_2]^{2+}$.

Magnetic parameters for the copper(II) complexes with L^{AX} are reported in Table 3, together with those associated with the bis complexes of histamine and L. They have been obtained by carefully simulating the experimental EPR spectra on the basis of the well known spin Hamiltonian for $S = \frac{1}{2}$ systems, including terms for the hyperfine and superhyperfine interactions. In the case of the complexes of the AC and AD regioisomers a rhombic in-plane component in g not exceeding 0.03 was taken into account in order to obtain a reasonable fit. However, since this could be an artefact of the simulation program, only average values are reported in Table 3, which also contains the results of the UV/VIS measurements.

Moreover AOM ligand-field calculations were carried out to provide an indication of the kind of distortion undergone by the complex metal sites when copper is forced to co-ordinate to two molecules of histamine covalently attached to the different glucose units of the cyclodextrin cavity. The fit was also extended to the electronic transitions (comparing the calculated transitions with those obtained from a deconvolution of the experimental optical spectra) to have more parameters to deal with. These calculations showed that the three different metal sites are distorted when compared with that of the bis complex with free histamine and this distortion involves both in- and out-of-plane angles. As shown in Table 4, the calculated magnetic parameters were obtained keeping the values of $e_{\sigma,\pi}(N)$ and $e_{\sigma,\pi}(O)$ constant and varying the in- and out-of-plane bonding angles as defined in Fig. 2. Variations of the Steven's orbital reduction factors k_{xy}, k_z were also made in order to account for strongly modified geometries. The copper(II) complex with L^{AB} showed the most distorted polyhedron. It was necessary to change both the in- and out-of-plane angles by several degrees in order to have a reasonable fit. The situation is a little different in the complex with the L^{AC} isomer. In this case a greater distortion of the in-plane angles accounts for a difficulty in accommodating copper due to a greater distance. It was also interesting that the copper(II) complex with L^{AD} showed the lowest $g_{\|}$ value, the least-distorted polyhedron, with out-of-plane angles closest to 90°. A less-distorted polyhedron leads to a more covalent bond situation. This could imply that the copper co-ordination of the four nitrogen donor atoms of the two molecules of histamine, covalently attached to opposite positions in the cyclodextrin ring, induced a perturbation of the symmetry of the cavity. In other words, the co-ordination of the copper to L^{AD} causes some of the cyclodextrin hydrogen bonds to weaken at the wider rim. As a consequence, the resulting complex could be more symmetric than the analogous ones with L^{AB} or L^{AC}. These evident differences within the copper(II) complex stereochemistries, deduced from the EPR and electronic spectra, could explain some of the differences found in their catalytic behaviour against O_2^- and will be discussed later.

Table 5 reports the SOD-like activity of the copper(II) complexes of the three isomers determined by the indirect assay using cytochrome c, and for comparison those pertinent to SOD and $[Cu(HPO_4)]$, which are in good agreement with data previously reported.[9,10,25] We may assume that the three regioisomer ligands form copper(II) complexes of similar stability. Hence we have used an excess of ligand (5:1) in order to minimize the contribution of the other complex species with respect to $[CuL^{AX}]^{2+}$ under the experimental conditions of the assay. These three SOD analogues display considerable superoxide dismutase-mimicking activity. In the case of $[CuL^{AB}]^{2+}$ the activity is about 10 times higher than that of $[Cu(HPO_4)]$. The other two complexes also have I_{50} values lower than that of $[Cu(HPO_4)]$. These difunctionalized cyclodextrins have four nitrogens which complex the copper(II), similar to the native enzyme, and thus it is not unusual that they are good SOD enzyme models. Furthermore, they have a larger stability constant in comparison with those of other copper(II) complexes previously investigated.[9,10] Unlike the complexes of 6-(5-amino-3-azapentylamino)-6-deoxy-β-cyclodextrin or glycylhistidyllysinate,[9] where the strong equatorial field probably determines their low scavenger ability, thus disfavouring axial binding of O_2^-, in the case of $[CuL^{AX}]^{2+}$ a higher SOD-like activity was found. As shown by the AOM results, the flexible co-ordination geometry of the two histamine chains can stabilize copper(I) by use of a tetrahedral geometry.

A suitable model system for testing the SOD-like activity of

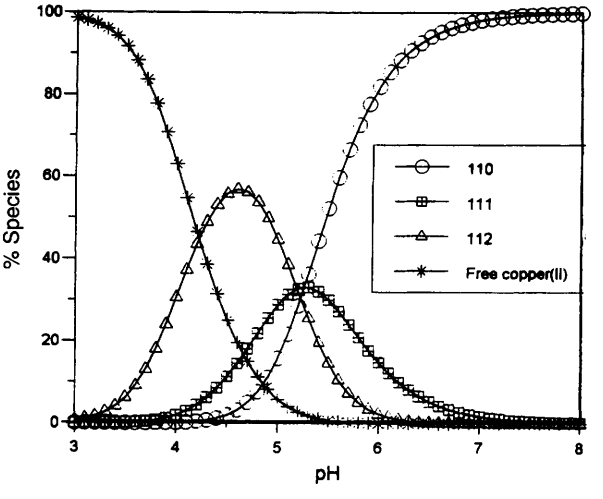

Fig. 1 Plot of percentage of copper(II) species in aqueous solution; $[Cu] = [L^{AB}] = 5 \times 10^{-3}$ mol dm^{-3}

Table 2 Stability constants of L^{AB} complexes with copper(II) at 25 °C and 0.10 mol dm^{-3} (KNO_3)

Equilibrium	log K	Ref.
$Cu^{2+} + hist \rightleftharpoons [Cu(hist)]^{2+}$	9.57	24
$[Cu(hist)]^{2+} + hist \rightleftharpoons [Cu(hist)_2]^{2+}$	6.56	24
$Cu^{2+} + [Hhist]^{+} \rightleftharpoons [Cu(Hhist)]^{3+}$	3.07	24
$Cu^{2+} + L \rightleftharpoons [CuL]^{2+}$	7.26	24
$Cu^{2+} + [HL]^{+} \rightleftharpoons [Cu(HL)]^{3+}$	2.94	24
$Cu^{2+} + L^{AB} \rightleftharpoons [CuL^{AB}]^{2+}$	10.11(2)	This work
$Cu^{2+} + [HL^{AB}]^{+} \rightleftharpoons [Cu(HL^{AB})]^{3+}$	7.62(1)	This work
$Cu^{2+} + [H_2L^{AB}]^{2+} \rightleftharpoons [Cu(H_2L^{AB})]^{4+}$	5.19(1)	This work

Table 3 Spin-Hamiltonian parameters obtained from the computer simulation of frozen-solution EPR spectra of copper(II) complexes with L^{AX} in water–methanol (9:1) at 150 K

Complex	$g_{\|}$	$A_{\|}$/cm^{-1}	$g_{\perp}$	$A_{\perp}$/cm^{-1}	$\tilde{\nu}$/cm^{-1}	ε_{max}/dm^3 mol^{-1} cm^{-1}
$[Cu(hist)_2]^{2+}$	2.231(2)	0.019 4(2)	2.052(5)	0.002 3(5)	16 722(70)	34
$[CuL_2]^{2+}$	2.246(2)	0.019 0(2)	2.061(5)	0.002 0(5)	15 408(70)	40
$[CuL^{AB}]^{2+}$	2.255(2)	0.018 3(2)	2.065(5)	0.002 5(5)	15 674(70)	84
$[CuL^{AC}]^{2+}$	2.255(2)	0.018 2(2)	2.075(5)	0.002 3(5)	15 823(70)	78
$[CuL^{AD}]^{2+}$	2.242(2)	0.018 1(2)	2.065(5)	0.002 3(5)	16 026(70)	76

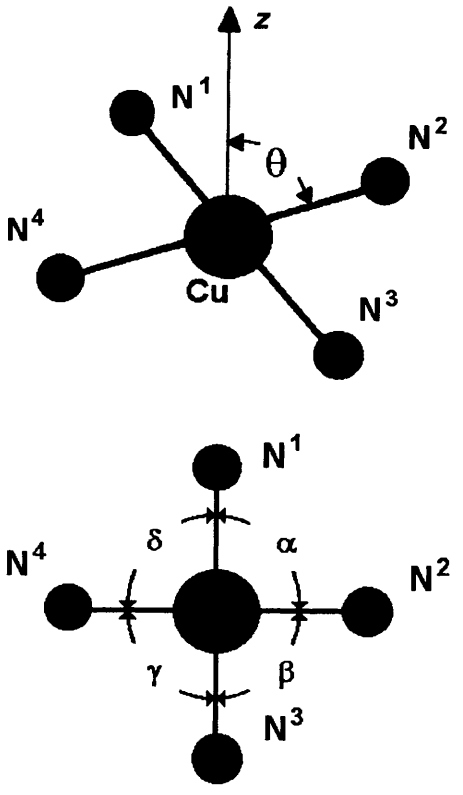

Fig. 2 Sketches of the copper(II) co-ordination site in the complexes with L^{AX} ligands showing the relevant bond angles used in the AOM calculations

Table 4 Results from AOM calculations with reference to the angles defined in Fig. 1 and by using the following constant parameters: $\lambda = -828$ cm^{-1}, $P = 0.0305$ cm^{-1}, $k = 0.43$, $e_\sigma(N^{1-4}) = 6000$ cm^{-1}, $e_\sigma(N^{2-3}) = 5900$ cm^{-1}, $e_\pi(N^{2-3}) = 1400$ cm^{-1}, $e_\sigma(O_w) = 1600$ cm^{-1}, $e_\pi(O_w) = 500$ cm^{-1}

	$[Cu(hist)_2]^{2+}$	$[CuL^{AB}]^{2+}$	$[CuL^{AC}]^{2+}$	$[CuL^{AD}]^{2+}$
$g_\|$	2.230	2.253	2.255	2.242
$A_\|$/cm^{-1}	0.019 2	0.018 4	0.018 2	0.018 2
$g_\perp$	2.056	2.066	2.071	2.067
$A_\perp$/cm^{-1}	0.003 2	0.003 2	0.003 3	0.003 2
$\tilde{\nu}_1$[a]/cm^{-1}	18 002	16 392	16 461	16 997
$\tilde{\nu}_2$[a]/cm^{-1}	16 395	14 572	14 950	15 566
$\tilde{\nu}_3$[a]/cm^{-1}	15 576	13 556	13 979	14 669
$\theta(N^1)$[b]/°	90	82	85	88
$\theta(N^2)$[b]/°	90	98	95	92
$\theta(N^3)$[b]/°	90	82	85	88
$\theta(N^4)$[b]/°	90	98	95	92
α/°	90	78	76	78
β/°	90	102	104	102
γ/°	90	78	76	78
δ/°	90	102	104	102
K_{xy}	0.760	0.780	0.830	0.805
K_z	0.800	0.778	0.772	0.773

[a] Calculated electronic transitions differed from deconvoluted spectra by not more than 100 cm^{-1}. [b] Atoms N^1 and N^4 are histamine amine nitrogens, N^2 and N^3 histamine imidazole nitrogens, and O_w the oxygen atoms of water molecules apically linked to copper.

these complexes is also provided by photosensitization systems in which a biological substrate is investigated for its resistance to transient or stable damaging species generated by an exogenous photosensitizer, when exposed to light of suitable intensity and wavelength. In this case we have used ketoprofen which is a well known photosensitizer and was previously shown to induce haemolysis and lipid peroxidation *via* type I (radical-like) and II (singlet oxygen) mechanisms.[1,22] We observed that these complexes protect in a dose-dependent way

Table 5 The SOD-like activity of copper(II) complexes with L^{AX} (X = B, C, or D)

Compound	$10^7 I_{50}$*/mol dm^{-3}
SOD	0.15 ± 0.01
$[Cu(HPO_4)]$	9.0 ± 0.04
$[CuL^{AB}]^{2+}$	1.2 ± 0.06
$[CuL^{AC}]^{2+}$	1.8 ± 0.09
$[CuL^{AD}]^{2+}$	3.0 ± 0.15

* Amount of copper compound required for 50% inhibition of cytochrome c reduction. Each value with ± designation is the mean ± standard deviation of three experiments.

(Fig. 3) and their relative efficiency was compared by use of the value of F at the same concentration of 0.1 μmol dm^{-3} (Table 6). The proligand was used in suitable excess (L:M = 2:1) to form about 80% of the $[CuL^{AX}]^{2+}$ complex, assuming again a similar stability constant for the copper(II) complexes of the three regioisomers. A higher L:M ratio was not used, so as to avoid any influence of the free proligands on the observed photohaemolysis rate (mainly a thermal lytic activity towards the membrane). For a useful comparison, the protective activity of $[Cu(HPO_4)]$ and SOD enzyme are reported. It is also important to outline that these functionalized cds alone do not reduce the photosensitization process induced by ketoprofen, as previously observed for unfunctionalized cds.[22] This process can be excluded because this kind of protective action is directly correlated to an inclusion process which can be disregarded at this concentration.[25] If we compare the I_{50} and the F values of the investigated complexes there is a good correlation, at least as regards the relative positions in the scale of activities. Some differences in the activity ratio (for example the high activity of the L^{AB} derivative with respect to the L^{AC} and L^{AD} compounds, which is much more evident in the red blood cell model than in the enzymatic assay) can be explained by the slight differences in the experimental conditions of the two assays. However, the experimental results undoubtedly show that in the described photoprotective device a SOD-mimicking mechanism is operative.

The copper(II) complex with the L^{AD} regioisomer gave the highest I_{50} in the enzymatic test, and thus showed the lowest catalytic activity. In addition, it differed by at least a factor of five from the analogous complex with L^{AB} in the photohaemolysis experiments. These findings parallel the results obtained by EPR and optical spectroscopies. As previously discussed, this copper(II) complex is the least distorted and, probably, the most stable in the series with L^{AX}, due to the more symmetric disposition of the histamine moieties on the cyclodextrin ring. On the contrary, the complex with L^{AB} showed the highest catalytic activity against O_2^- and this fact could be certainly ascribed to the greater tetrahedral distortion, which is the discriminating factor among these complexes. It is well known that copper(I) species prefer tetrahedral or linear co-ordination. The copper(II) complex with L^{AC} showed an intermediate behaviour, which is closer to the analogous complex with L^{AB}, in the enzymatic test, and closer to L^{AD} in the photohaemolysis experiments.

Conclusion

An active-site analogue of Cu_2Zn_2-SOD should co-ordinate copper(II) as well as copper(I) ions. During the catalytic process the tetrahedrally distorted square-planar arrangement of SOD nitrogens (four histidine moieties in a more or less hydrophobic region) around the Cu^{II} stabilizes the copper(I) complex formation. Four nitrogen atoms are provided by these difunctionalized cyclodextrins. The synthesis of the different regioisomers allows the modulation of the stability, geometry and other bonding features of the copper(II) complexes

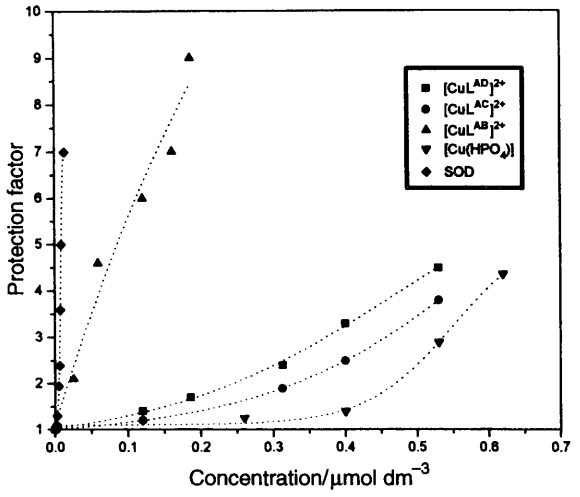

Fig. 3 Dose-dependent protective activity of the complex species existing under the experimental conditions of the ketoprofen photohaemolysis assay; 4×10^{-5} mol dm^{-3} ketoprofen, 3.3×10^{6} red blood cells cm^{-3}, $T = 20$ °C, irradiation time 5 or 10 min. Each point is the mean ± standard deviation of triplicate experiments; Boltzmann sigmoidal fitting has been applied

Table 6 Protective activity of the complexed species existing under the experimental conditions of the ketoprofen induced photohaemolysis

Complex	F at 0.1 μmol dm^{-3}
[CuL^{AB}]$^{2+}$	5.50 ± 0.3
[CuL^{AC}]$^{2+}$	1.15 ± 0.07
[CuL^{AD}]$^{2+}$	1.30 ± 0.08
[Cu(HPO$_4$)]	1.13 ± 0.07
SOD	> 24

Each value with ± designation is the mean ± standard deviation of three experiments. Boltzmann sigmoidal fitting has been applied.

(equatorial field strength, axial water interaction, *etc.*). It is possible to conclude that the peculiar geometry of the copper(II) complex is the discriminating factor. On the basis of AOM calculations, it was shown that the copper(II) complex with L^{AB} has a co-ordination polyhedron which is more distorted towards a tetrahedral arrangement. It is also important to consider that the proximity of the two molecules of histamine gives a certain flexibility to this metal site, which could easily balance the stereochemical changes due to the reduction to copper(I).

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