Superoxide Dismutase Activity of $Cu(II)_2(aspirinate)_4$ and Its Adducts with Nitrogen and Oxygen Donors

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Abstract

Several adducts of $Cu(II)_2(aspirinate)_4$ of formula $[Cu(II)(aspirinate)_2L_2]$, where L is pyridine, nicotinamide, 3-picoline, 4-picoline, imidazole, 1-methylimidazole, diethylamine and dimethyl sulfoxide, have been synthesized. They show an absorption wavelength maximum between 690 and 750 nm, which is assigned to the d–d transition. The infrared spectra of these adducts indicate the bonding of nitrogen to copper in their nitrogen base adducts and the bonding of oxygen to copper in the DMSO adduct. The frozen ESR spectra of the adducts suggest that the ligands are bonded in an axial environment around copper with two nitrogen donors occupying the equatorial plane. The lower $g_{\|}$ value of the DMSO adduct as compared to $g_{\|}$ values of the nitrogen base adducts implies that there is bonding of the oxygen atom of DMSO in its adduct. The superoxide dismutase activity of several of these adducts has been measured. The pyridine adduct of $Cu(II)_2(aspitinate)_4$ shows a much higher activity than its DMSO adduct. This is also reflected in the enhanced antitumor activity of the pyridine adduct over the DMSO adduct of $Cu(II)_2(aspirinate)_4$.

Introduction

Copper complexes have shown antiinflammatory, antiulcer, anticonvulsant, antidiabetic, anticancer, anticarcinogenic, antimutagenic and radioprotectant activities in animal models of diseased states [1–5]. In 1979, Oberley and Buettner observed that cancer cells had less superoxide dismutase activity than normal cells [6]. This has led to the successful testing of $Cu(II)_2(aspirinate)_4$ and copper(II) salicylates against the growth of a solid tumor in implanted mice. Subsequently, several copper amino acid, peptide and other complexes have been found to be effective in the same or similar animal tumor models as well as in tumor cell cultures [7–15]. The antitumor activity of $Cu(II)_2(aspirinate)_4$ is enhanced in its more soluble monomeric adduct of pyridine [6]. The antitumor activity of these complexes has been suggested to be due to their superoxide scavenging ability [3]. Therefore, we report here the synthesis, spectroscopic studies and superoxide dismutase activity of the adducts of $Cu(II)_2(aspirinate)_4$ with pyridine, nicotinamide, picolines, imidazoles and dimethyl sulfoxide (DMSO).

Experimental

Starting Materials

Copper(II) chloride ($CuCl_2 \cdot 2H_2O$), nitro blue tetrazolium chloride monohydrate (NBT), nicotinamide (niam), DMSO, pyridine (py) and diethylamine (deam) were brought from S.D. Fine-chem. (India). Imidazole (imH), 4-picoline (4-pic) and 3-picoline (3-pic) were purchased from BDH (U.K.), and 1-methylimidazole (1-Meim) was obtained from Aldrich (U.S.A.). Acetylsalicylic acid (aspirin) and superoxide dismutase from bovine blood were bought from Sigma (U.S.A.). Other chemicals used were of analytical reagent grade. Solvents used were purified before use by the standard methods [16].

Synthetic Procedures

$[Cu(II)_2(aspirinate)_4]$ (a) [17]

Acetylsalicylic acid (30 g) was dissolved in 200 ml of water at 0 °C with 50% sodium hydroxide (wt./vol.); the final pH of this solution was about 8. A solution of 56 g of $CuCl_2 \cdot 2H_2O$ in 500 ml was added over 10 min to the above solution. The blue precipitate obtained was washed with water and acetone. The complex was dried in a vacuum desiccator over anhydrous calcium chloride.

$[Cu(II)(aspirinate)_2(py)_2]$ (b)

This complex was prepared by a modification of the method described earlier [17].

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To a suspension of $Cu(II)_2(aspirinate)_4$ (5 mmol) in 80 ml of benzene or chloroform, pyridine (25 mmol) was added with stirring. The mixture was stirred for 3 h. The dark blue clear solution was filtered and concentrated at 28 °C. The blue needle like crystals were collected by filtration, washed with cold benzene and dried in a vacuum desiccator over anhydrous calcium chloride.

[Cu(II)(aspirinate)$_2$(3-pic)$_2$] (c)

This complex was prepared by the method described for **b** except that 3-picoline (25 mmol) was used in place of pyridine.

[Cu(II)(aspirinate)$_2$(4-pic)$_2$] (d)

This complex was prepared by the method described for **b** except that 4-picoline (25 mmol) was used in place of pyridine.

[Cu(II)(aspirinate)$_2$(imH)$_2$] (e)

Imidazole (25 mmol) was dissolved in 80 ml of dioxane containing 10 ml of methanol. To the above stirred solution 5 mmol of $Cu(II)_2(aspirinate)_4$ was added. The solution was stirred for half an hour. The clear blue solution was filtered and concentrated to a small volume. The blue crystals were collected by filtration, recrystallized from benzene, and dried in a vacuum desiccator over anhydrous calcium chloride.

[Cu(II)(aspirinate)$_2$(1-Meim)$_2$] (f)

This complex was prepared by the method described for **e** except that 1-methylimidazole (25 mmol) was used in place of imidazole.

[Cu(II)(aspirinate)$_2$(deam)$_2$] (g)

This complex was prepared by the method described for **b** except that diethylamine (25 mmol) was used in place of pyridine.

[Cu(II)(aspirinate)$_2$(niam)$_2$] (h)

Nicotinamide (25 mmol) was dissolved in 80 ml of dioxane. The solution was heated at 70 °C followed by addition of 5 mmol of $[Cu(II)_2(aspirinate)_4]$. The mixture was stirred for 1 h. After stirring the bluish green solution was filtered and the filtrate was treated with diethyl ether. The green crystals obtained were collected by filtration, washed with diethyl ether and recrystallized from dioxane. The product was dried in a vacuum desiccator over anhydrous calcium chloride.

[Cu(II)(aspirinate)$_2$(DMSO)$_2$] (i)

The complex was prepared by dissolving $[Cu(II)_2(aspirinate)_4]$ in hot dimethyl sulfoxide. The hot solution was stirred for half an hour and then filtered. On cooling the filtrate, the greenish complex crystallized out. The product was collected by filtration, washed with diethyl ether, and dried in a vacuum desiccator over anhydrous calcium chloride.

Chemical analysis of the above complexes was performed at the Microanalytical Laboratory, I.I.T., Bombay.

Physical Measurements

The electronic absorption spectra of the complexes in aqueous solution were recorded using Varian Superscan-3 and Shimadzu-260 spectrophotometers. The infrared spectra of the complexes were recorded using Perkin-Elmer-237 spectrophotometer and Nicolet-170 SX FTIR spectrometer (4000 to 400 cm^{-1}) in KBr pellet or Nujol mull. The electron spin resonance (ESR) spectra of the complexes in solution at 25 °C and in frozen solid at liquid nitrogen temperature (77 K) were recorded on a Varian-112 ESR spectrometer using tetracyanoethylene (TCNE) as g marker ($g = 2.00277$). The frozen solutions at liquid nitrogen temperature used for ESR spectra were either in chloroform or in methanol. The room temperature solution spectra of the complexes were measured using Varian aqueous solution cell (E-248) and Varian quartz tubes were employed for taking ESR spectra of powder and frozen solutions. The ESR parameters for copper(II) complexes at 77 K were determined accurately from the Monte Carlo Computer simulation program [18].

Alkaline Dimethyl Sulfoxide–Nitro Blue Tetrazolium Chloride

An assay using alkaline dimethyl sulfoxide as a source of superoxide ion [19] and nitro blue tetrazolium chloride (NBT) as a scavenger of superoxide ion was performed as follows. A typical 400 μl sample to be assayed was added to a solution containing 2.1 ml of 0.2 M potassium phosphate buffer (pH 8.6) and 1 ml of 56 μm NBT. The tubes were kept in ice for 15 min. Then 1.5 ml of superoxide solution (alkaline DMSO) was added with stirring. The absorbance of the violet color developed was measured at 560 nm against a sample prepared under similar conditions except NaOH was absent in DMSO. A unit of superoxide dismutase (SOD) activity is defined as the amount of complex or enzyme which causes 50% inhibition of NBT reduction [19].

Results and Discussion

The adducts of $Cu(II)_2(aspirinate)_4$ with pyridine, nicotinamide, 3-picoline, 4-picoline, imidazole, 1-methylimidazole, diethylamine and dimethyl sulfoxide have been prepared and the chemical analyses are given in Table 1.

The above adducts show one broad absorption band in the visible region (see Table 1), which is assigned to the d–d transition. The absorption maxima for dimethyl sulfoxide adducts is higher than the nitrogen donor adducts of $[Cu(II)_2(aspirinate)_4]$.

TABLE 1. Elemental analysis and visible absorption data of copper(II) complexes

Complex	Found (calculated) (%)			λ_{max} (nm) (ϵ_{max})[a]	
	C	H	N	H_2O	CH_3OH
a	51.41 (51.25)	3.4 (3.32)			
b	58.70 (57.98)	4.23 (4.14)	4.67 (4.83)	739 (30)	740 (62.5)
c	59.13 (59.26)	5.00 (4.61)	4.67 (4.61)	695 (45)	
d	59.60 (59.26)	4.70 (4.61)	5.00 (4.61)	705 (51)	730 (75)
e	51.61 (52.03)	3.70 (3.25)	10.00 (10.12)	698 (47)	690 (75)
f	53.41 (53.65)	4.40 (3.79)	9.90 (9.63)	697 (55)	692 (70)
g	52.00 (51.81)	4.90 (5.10)	5.70 (5.50)		730 (68)
h	54.20 (54.09)	4.00 (3.91)	8.20 (8.41)		740 (70)
i	51.60 (51.41)	4.95 (5.06)		781 (20)	740 (69)

[a] ϵ_{max} is the molar extinction coefficient at wavelength maximum (λ_{max}) in 1 mol^{-1} cm^{-1}.

The coordination of the nitrogen donor introduces a stronger ligand field than the oxygen donors, which results in the shifting of absorption maxima to shorter wavelengths for the nitrogen adducts [20]. The absorption maxima for pyridine and 4-picoline adducts are higher than the absorption maximum of the 3-picoline adduct. This suggests that 3-pyridine exerts a stronger ligand field than pyridine and 4-picoline. The imidazole and 1-methylimidazole adducts show lower wavelength absorption maxima than pyridine and related adducts. This may be due to imidazole behaving as a stronger σ-donor than pyridine thereby showing a blue shift in their absorption maxima [18, 19]. The crystal structure analysis of the pyridine adduct shows that the copper atom is bonded in a *trans* square planar arrangement to the nitrogen atom of two pyridine molecules and one carboxylate oxygen atom from each of two aspirinate anions [21].

The infrared spectrum of $Cu(II)_2(aspirinate)_4$ shows two different stretching vibrations at 1750 and 1720 cm^{-1} due to two types of acetoxy carbonyl groups as revealed by X-ray structural analysis [22]. One of these is weakly bonded to a copper atom of a neighbouring $Cu(II)_2(aspirinate)_4$ molecule, while the other is not. The infrared absorption spectrum of this complex also contains a single antisymmetric carboxylate stretching vibration at 1610 cm^{-1}. The pyridine adduct of $Cu(II)_2(aspirinate)_4$ shows an antisymmetric carboxylate stretching vibration at 1610 cm^{-1}. In addition, the infrared spectrum of this adduct shows bands at 1080, 1060 and 1010 cm^{-1}, which are characteristic of pyridine ring vibrations. These bands are shifted towards a higher frequency as compared to the corresponding bands of non-bonded pyridine, which occur at 1057, 1029 and 989 cm^{-1}. The band at 1748 cm^{-1} has been assigned to the carbonyl stretching vibration [21, 23]. The observed single carbonyl stretching vibration for the acetoxy group is consistent with the X-ray crystallographic data for the pyridine adduct which shows only one type of acetoxy group. The crystal structure of this pyridine adduct indicates that a single copper atom is bonded to the nitrogen atom of two pyridine molecules in a *trans* square planar arrangement and to one oxygen atom of carboxylate from each of the two aspirinate anions. The other carboxylate atoms are weakly bonded to copper and the direction of the Cu–O bonds lies at 34.8° from the normal to the CuO_2N_2 plane [21]. The 3-picoline and 4-picoline adducts of $Cu(II)_2(aspirinate)_4$ show a carboxylate stretching vibration at 1610 cm^{-1} and a carbonyl stretching vibration at 1760 cm^{-1}. These complexes also show one carbonyl stretching vibration of the acetoxy carbonyl groups of aspirin indicating the same type of environment for both the acetoxy carbonyl groups. On the basis of carbonyl stretching vibrations, one could expect the same structures for the 3-picoline and 4-picoline adducts with the corresponding pyridine adduct.

The imidazole and 1-methylimidazole adducts of $Cu(II)_2(aspirinate)_4$ show carboxylate stretching vibrations at 1600 and 1615 cm^{-1}, respectively. In addition, both adducts show two strong bands in the region of 1750 to 1775 cm^{-1} due to stretching vibrations of the carbonyl groups of aspirin. This suggests that the two types of acetoxy carbonyl groups are present in these complexes. Two types of acetoxy groups are also present in $Cu(II)_2(aspirinate)_4$ [22]. The nicotinamide adduct of $Cu(II)_2(aspirinate)_4$ shows the binding of the heterocyclic nitrogen as there is no change in the amide NH stretching vibrations (3360 and 3200 cm^{-1}). In addition, the bands at 1635 and 1665 cm^{-1} due to the carbonyl frequencies of amide are also not affected after complex formation [24], and the acetoxy carbonyl group shows a stretching vibration at 1750 cm^{-1}. The dimethyl sulfoxide adduct of $Cu(II)_2(aspirinate)_4$ shows a S–O stretching vibration at 1010 cm^{-1}. This frequency is lower than that for free DMSO (1100–1055 cm^{-1}) and thus indicates that the binding of DMSO is through oxygen [23].

The ESR parameters, g and A tensors, for these complexes are given in Table 2. A typical frozen solution ESR spectrum of [Cu(II)(aspirinate)$_2$-

TABLE 2. ESR parameters of copper(II) complexes

Complex	$g_\parallel$	$g_\perp$	g_0	$A_\parallel$ [a]	$A_\perp$ [a]	A_0 [a]	A_N [a]
a[b]			2.077				
b[c]	2.283	2.098	2.16	178	6	61	15
c[c]	2.272	2.067	2.16	186	3	61	
d[c]	2.281	2.099	2.16	181	5	61	15
e[d]	2.272	2.063	2.158	175	19	71	16
f[d]	2.275	2.084	2.153	168	12	60	15
g[c]	2.280	2.087	2.16	183	4	62	15
h[c]	2.298	2.097	2.164	172	3	56	15
i[c]	2.412	2.076	2.185	141	19	56	

[a] $(cm^{-1}) \times 10^4$. [b] Powder spectrum. [c] In chloroform at 77 K and 25 °C. [d] In methanol at 77 K and 25 °C.

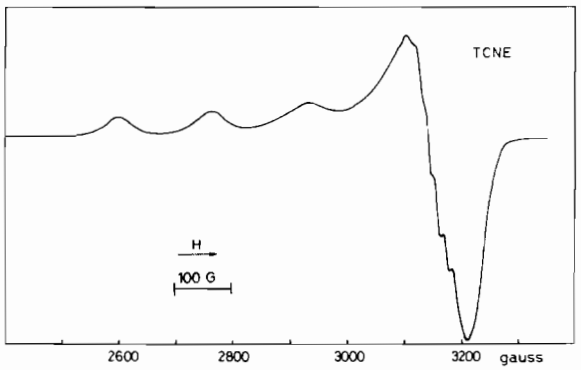

Fig. 1. Frozen methanol solution ESR spectrum of Cu(II)-(aspirinate)$_2$(imH)$_2$ (~10^{-3} M) at 77 K.

$(imH)_2$] (e) is given in Fig. 1. These complexes show a four line isotropic solution spectra at 25 °C with the isotropic g_0 value of about 2.16. The DMSO adduct (i) shows a higher g_0 value of 2.185. This is expected because of its lower ligand field strength compared to the nitrogen donors. The ESR spectra of the complexes at 77 K are indicative of an axial environment around copper(II). Such an environment produces a pattern of four lines in the $g_\parallel$ region and a strong signal in the $g_\perp$ region (see Fig. 1). The spectra of complexes containing nitrogen donors show five line superhyperfine structure in the $g_\perp$ region. The five superfine lines indicate the presence of two nitrogen donors in the equatorial plane of the first coordination sphere with the ground state of the complexes being $d_{x^2-y^2}$ [25, 26]. This is in good agreement with the observed crystal structure of the pyridine adduct (b) [21].

Adducts of the nitrogen donors show lower $g_\parallel$ values than the DMSO adduct (i). The infrared and visible absorption spectra of the DMSO adduct (i) indicate bonding of DMSO through oxygen. The highest λ_{max} value of the DMSO adduct (i) in the series (see Table 1) indicates the weak equatorial ligand field. Thus the ligand field exerted by DMSO in its adduct (i) is lower than the nitrogen donors in their adducts. This is responsible for a higher $g_\parallel$ value in the DMSO adduct (i) than in the nitrogen donor adducts.

The imidazole, 1-methylimidazole and 3-picoline adducts have lower $g_\parallel$ values than the pyridine and 4-picoline adducts. These results are consistent with the visible absorption spectral data which show lower λ_{max} values for imidazole, 1-methylimidazole and 3-picoline adducts than for the pyridine and 4-picoline adducts. This can be explained on the basis of the stronger σ-donation ability of imidazoles compared to pyridine [25].

The superoxide dismutase activity of several copper(II) complexes has been measured and the data are given in Table 3. The pyridine adduct of $Cu(II)_2$-$(aspirinate)_4$ (see Fig. 2) shows the highest activity whereas Cu(II)$(aspirinate)_2(DMSO)_2$ shows the lowest activity. The adducts given in Table 3 follow the activity order: pyridine > 1-methylimidazole > 4-picoline > imidazole > dimethyl sulfoxide. The adduct Cu(II)$(aspirinate)_2(py)_2$ shows about 3 times

TABLE 3. Superoxide dismutase activity of copper(II) complexes by NBT method

Complex	Amount required for unit SOD activity (μM)
Cu(II)$(aspirinate)_2(DMSO)_2$	>400
Cu(II)$(aspirinate)_2(imH)_2$	160
Cu(II)$(aspirinate)_2(4\text{-}pic)_2$	100
Cu(II)$(aspirinate)_2(1\text{-}Meim)_2$	38
Cu(II)$(aspirinate)_2(py)_2$	13
Cu(II)$(salicylate)_2$	44
Superoxide dismutase	0.72

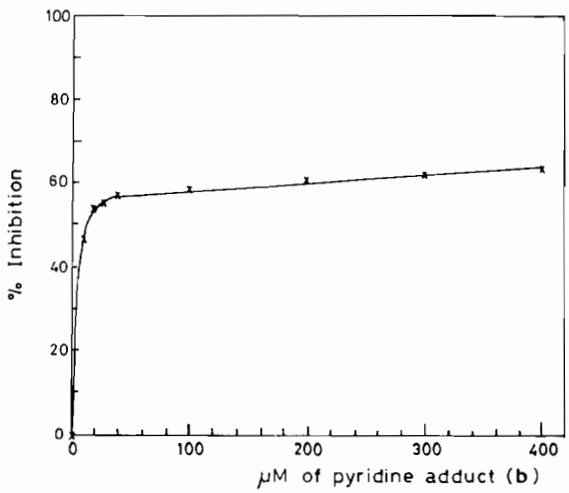

Fig. 2. A plot of percent inhibition with increase in concentration of Cu(II)$(aspirinate)_2(py)_2$ (0–400 μM).

more activity than Cu(II)(salicylate)$_2$ and 18 times less activity than the native enzyme on a molar basis. On the other hand the least active adduct Cu(II)-(aspirinate)$_2$(DMSO)$_2$ is about 10 times less active than Cu(II)(salicylate)$_2$ and about 600 times less active than the native enzyme on a molar basis. The much higher activity of the pyridine adduct of Cu(II)$_2$(aspirinate)$_4$ compared to the DMSO adduct is also reflected in the enhanced antitumor activity of the pyridine adduct over the DMSO adduct [6].

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