Inorganica Chimica Acta, *125* (1986) 117–121

The Di-Schiff-base of Pyridine-2-aldehyde and 1,4-Diaminobutane, a Flexible Cu(I)/Cu(II) Chelator of Significant Superoxide Dismutase Mimetic Activity[*]

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(Received April 3, 1986)

Abstract

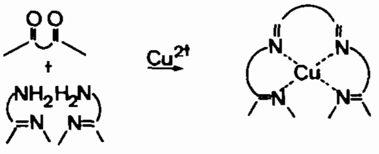
It was attempted to devise a stable and flexible chelator capable to coordinate both Cu(I) and Cu(II) and which would be catalytically active to accelerate superoxide dismutation. As in Cu_2Zn_2-superoxide dismutase, the chelating ligands around the copper were expected to be distorted square planar for Cu(II) and tetrahedral in the case of Cu(I). The di-Schiff-base of pyridine-2-aldehyde and 1,4-diamino-butane turned out to be a tetradentate chelate and fulfilled these structural criteria quite well. The electronic absorption at 710 nm and a $g_{\parallel}/A_{\parallel}$-value of 134 cm are indicative for the beginning of a tetrahedral distortion. These data are in close proximity to those obtained with Cu_2Zn_2-superoxide dismutase. The superoxide dismutase mimetic activity was 3% of that of the native enzyme and was 60 times higher compared to the activity of copper complexes using amino acids or salicylates.

Introduction

Cu_2Zn_2superoxide dismutase catalyses the proton dependent dismutation of superoxide into dioxygen and hydrogen peroxide [1]. During this reaction the copper undergoes a redox change from Cu(II) into Cu(I) [2]. In the active site of the cupric enzyme the Cu(II) is centred in a distorted square planar arrangement coordinated to the nitrogen atoms of his-44, -46, -61 and -118, respectively. His-61 serves as the bridging ligand to the Zn(II) binding site. There, his-69 and -78 provide two more imidazolate nitrogens and the oxygen of the carboxylate group of asp-81 acts as the fourth ligand to the zinc while a tetrahedral structure with a strong distortion towards a trigonal pyramid [3, 4] is maintained. Throughout the catalytic cycle the copper is the most important metal and remains firmly bound in the active centre. The geometry varies from tetrahedral in the case of Cu(I) to the above mentioned distorted square planar arrangement of the Cu(II). The protein moiety is flexible enough to allow these geometric changes without losing the metal. Unlike the native enzyme, there are low M_r Cu–complexes where Cu(II) is coordinated in an acetate type structure [5] or as simple amino acid chelates [6]. According to pulse radiolysis measurements these Cu–complexes display identical rate constants compared to that of Cu_2Zn_2superoxide dismutase [7]. However, the SOD mimicking compounds are not stable in the Cu(I) form. Both geometry and the first shell atoms around the transiently formed Cu(I)-compounds are poor substitutes for the active centre of the native enzyme. Thus, it was attempted to devise and prepare flexible mononuclear Cu–chelates which will be stable in the +I and +II form. Furthermore, they should be efficient catalysts for superoxide dismutation.

Suitable ligands which were thought to fulfill these criteria promised to be di-Schiff-bases prepared from either dicarbonylic compounds (aldehyde or ketone) (Scheme 1) and a primary amine or by the condensation of an aromatic *N*-heterocyclic aldehyde and an aliphatic diamine (Scheme 2).

The preparation of some of these Cu–complexes are described. Physicochemical properties including



Scheme 1. Reaction of dicarbonylate with a primary amine.

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[*] Abbreviations used: **I**, $CuL_{py}ClO_4$: {3-(2-pyridylethyl-imino)[(1,3-diphenyl)prop-1-enonato]-(*N*, *N*′, *O*)} copper(II), perchlorate; **II**, $CuL_{py}NO_3$: {3-(2-pyridylethyl-imino)[(1,3-diphenyl)prop-1-enonato]-(*N*, *N*′, *O*)}copper(II), nitrate; **III**, $CuL_{hm}ClO_4$: {3-(4-imidazolylethyl-imino)[1,3-diphenyl)-prop-1-enonato]-(*N*,*N*′,*O*)}copper(II), perchlorate; **IV**, $CuL_{pupy}(ClO_4)_2$: {[*N*,*N*′-bis(2-pyridylmethylene)-1,4-butanediamine]-(*N*,*N*′,*N*″,*N*‴)}copper(II), diperchlorate; Cu_2Zn_2-SOD: Cu_2Zn_2superoxide dismutase; Cu_2E_2-SOD: Zn-free Cu_2superoxide dismutase.

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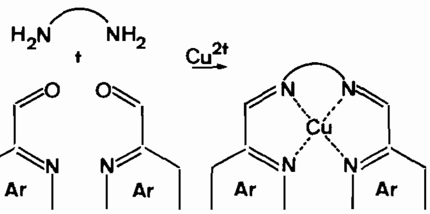


Scheme 2. Reaction of aromatic *N*-heterocyclic aldehyde with an aliphatic diamine.

infrared spectrometry, electronic absorption and magnetic measurements were compared with those of Cu_2Zn_2superoxide dismutase. Superoxide dismutase activity was monitored by the tetrazolium blue assay.

Experimental

Chemicals

2-(2-Aminoethyl)pyridine, 1,4-diaminobutane, dibenzoylmethane, pyridine-2-aldehyde and histamine dihydrochloride were from Ega-Chemie, Steinheim; xanthine, xanthine oxidase and nitrotetrazolium blue were from Serva, Heidelberg. Cu_2-Zn_2superoxide dismutase was prepared from bovine erythrocytes [8].

Complexes

{3-(2-Pyridylethyl-imino)[(1,3-diphenyl)prop-1-enonato]-(N,N′,O)}copper(II), perchlorate (I).

480 μl (4 mmol) 2-(2-Aminoethyl)pyridine and 740 mg (2 mmol) $Cu(ClO_4)_2 \cdot 6H_2O$ were dissolved in 30 ml ethanol. 448 mg (2 mmol) Dibenzoylmethane were added dropwise and at the end of the reaction refluxed for one hour. Upon cooling to 10 °C dark green crystals were obtained. They were washed with ethanol and allowed to dry in the presence of air. The yield was 730 mg (71.9%). *Anal.* Calc. for $C_{22}H_{21}ClCuN_2O_7$ (M_r 508.44): C, 51.96; H, 4.16; N, 5.51; Cu, 12.50. Found: C, 51.53; H, 4.15; N, 5.49; Cu, 12.87%.

{3-(2-Pyridylethyl-imino)[(1,3-diphenyl)prop-1-enonato]-(N,N′,O)}copper(II), perchlorate (I).

500 μl (4 mmol) 2-(2-Aminoethyl)pyridine and 480 mg (2 mmol) $Cu(NO_3)_2 \cdot 3H_2O$ were dissolved in 15 ml methanol. 448 mg (2 mmol) Dibenzoylmethane previously dissolved in 15 ml methanol were added dropwise at 22 °C. Stirring was continued for another hour. Excess solvent was removed under vacuum. The remaining green oil was dissolved in 10 ml CH_2Cl_2 and titrated with diethylether until turbidity was noticed. After 12 h dark green crystals precipitated at −20 °C. They were washed with −20 °C cold CH_2Cl_2 and kept under air to dry. The yield was 685 mg (72.7%). *Anal.* Calc. for $C_{22}H_{21}$-CuN_3O_6 (M_r 470.95): C, 51.10; H, 4.49; N, 8.92; Cu, 13.49. Found: C, 55.91; H, 4.37; N, 8.96; Cu, 13.87%.

{3-(4-Imidazolylethyl-imino)[1,3-diphenyl)prop-1-enonato]-(N,N′)}copper(II), perchlorate (III).

736 mg (4 mmol) Histamine dihydrochloride and 740 mg (2 mmol) $Cu(ClO_4)_2 \cdot 6H_2O$ were suspended in a mixture of 10 ml H_2O and 50 ml methanol. The solution was titrated with sodium carbonate to pH 9. 448 mg (2 mmol) Dibenzoylmethane in 15 ml methanol were added dropwise at 22 °C while stirring was maintained for more than one hour. After 3–5 days black–green crystals started to precipitate at −20 °C which were separated by filtration and washed with methanol, and recrystallized from methanol/water (80% *v*/*v*). The yield was 458 mg (46%). *Anal.* Calc. for $C_{20}H_{20}ClCuN_3$-O_5 (M_r 497.43): C, 48.29; H, 4.05; N, 8.45; Cu, 12.77. Found: C, 48.04; H, 3.99; N, 8.59; Cu, 13.08%.

{[N,N′-bis(2-Pyridylmethylene)-1,4-butanediamine]-(N,N′,N″,N‴)}copper(II), diperchlorate (IV).

Unlike an earlier synthesis using alcoholic solutions [9], a completely aqueous preparation method was devised and carried out. 380 μl (4 mmol) Pyridine-2-aldehyde and 740 mg (2 mmol) $Cu(ClO_4)_2 \cdot 6H_2O$ were dissolved in 10 ml H_2O. 3 ml Aqueous 1,4-diaminobutane (200 μl or 2 mmol) were added dropwise. Small turquoise blue needles precipitated within minutes. They were filtrated, washed with H_2O and ethanol. The yield was 705 mg (65.5%). *Anal.* Calc. for $C_{16}H_{19}Cl_2CuN_4O_{8.5}$ (M_r 537.9): C, 35.73; H, 3.56; N, 10.42; Cu, 11.81. Found: C, 35.50; H, 3.43; N, 10.39; Cu, 11.45%.

Spectrometry

Electronic absorption spectra were run on a Beckman spectrophotometer model 25. Electron paramagnetic resonance was measured on a Varian E 109 at modulation amplitude 10 G, modulation frequency 100 KHz, microwave power 10 mW, microwave frequency 9.24 GHz, temperature 77 K. Infrared spectroscopy was carried out using a Beckman spectrophotometer Acculab 4 and KBr disks. Elemental analyses of C, H, and N were performed on a Perkin-Elmer Elemental Analyzer 240 B. Copper was quantitated on a Perkin-Elmer atomic absorption spectrometer 400 S equipped with a graphite furnace HGA 76B.

Superoxide Dismutase Assay

Superoxide dismutase activity was assayed using the nitro blue tetrazolium assay [10]. 0.5 ml of the reaction volume contained 0.62 mM nitroblue tetrazolium, 20 mM HEPES buffer pH 7.4, 150 mM NaCl, 0.2% (*w*/*v*) gelatine, 50 μM xanthine, 0.18 μM xanthine oxidase, copper complexes and/or superoxide dismutase in variable concentrations. A_{540} was monitored in a 10 mm light path cell at 23 °C.

Results and Discussion

According to Scheme 1 it was expected that dicarbonylic compounds including aldehyde or ketones react with a primary amine. This amine should be bound to a nitrogen containing aromatic heterocycle. The resulting tetradentate ligand was expected to provide 4 unsaturated nitrogen atoms to allow coordination of both Cu(II) and Cu(I). The aromatic component should be conjugated with respect to the formed azomethine moiety to avoid hydrolysis of isolated C=N groups. Dibenzoylmethane served as the dicarbonyl compound and histamine or 2-(2-aminoethyl)pyridin were used as amines. A template reaction at the coordinated Cu(II) was carried out. Because of the strong delocalization of π-electrons in the dibenzoyl methane, which was even stronger in the copper-chelate ring, the reaction of the carbonyl group with the nucleophilic agents was substantially diminished. Thus, the second C=O moiety was unable to react with the amine. In the case of **I**, **II** and **III**, only mono-Schiff-bases were obtained (Figs. 1, 2). The second synthetic pathway using Cu(II) coordinated pyridine-2-aldehyde and 1,4-diaminobutane (Scheme 2) yielded the desired di-Schiff-bases. Unlike the former three complexes, this copper chelate proved to be quite soluble in aqueous systems, and, thus suitable for superoxide dismutase activity measurements (Fig. 3).

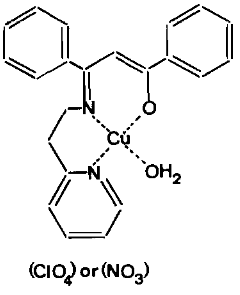


Fig. 1. **I**: $CuL_{py}(ClO_4)\cdot H_2O$, **II**: $CuL_{py}(NO_3)\cdot H_2O$.

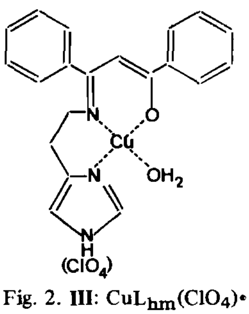


Fig. 2. **III**: $CuL_{hm}(ClO_4)\cdot H_2O$.

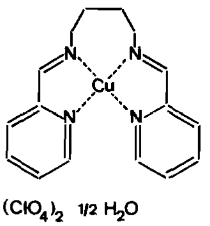
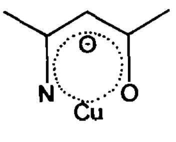


Fig. 3. **IV**: $CuL_{pupy}(ClO_4)_2\cdot 0.5H_2O$.

Infrared Spectroscopy

Infrared spectroscopy was performed to confirm whether or not the copper chelation was successful. In general the wavelength region between 1525–1590 cm^{-1} can be attributed to a 6-membered metal chelate [11].



In the absence of a ring structure $\nu_{C=N}$ would be completely leveled off and $\nu_{C=O}$ would appear at a much higher wavelength. It was important to notice that the ionic character of both NO_3^- and ClO_4^- remained unchanged, attributable to the strong bands at 1380 cm^{-1} and 1100 cm^{-1}, respectively. Thus, there was no direct coordination of these anions to the chelated Cu(II) (Table I). The azomethine group of **IV** is conjugated to the pyridine only with the consequence that $\nu_{C=N}$ is also seen at 1635 cm^{-1}. The infrared absorption at 1595 cm^{-1} is attributable to the aromatic C=C vibration.

Electron Paramagnetic Resonance

Electron paramagentic resonance measurements were carried out to monitor the chemical environment around the Cu(II) and to evaluate the relationship to the copper binding site of Cu_2Zn_2superoxide dismutase (Fig. 4, Table II). The ratio $g_{\parallel}/A_{\parallel}$ is an empirical factor for the tetrahedral distortion of a tetragonally arranged complex [12]. Square planar arrangements can be assigned between 105 and 135 cm. The progressive rise of up to 250 cm can be attributed to a tetrahedrally distorted arrangement. The complexes **I**–**III** are among those with the reported square planar environment. The $g_{\parallel}/A_{\parallel}$ value of 134 cm is indicative for the beginning of a tetrahedral distortion and is not too far away from the EPR properties of native Cu_2Zn_2superoxide dismutase [13].

TABLE I. Infrared Frequencies of Cu–Complexes I–IV in cm^{-1}

		ν_{N-H}	$\nu_{C=N}$	$\nu_{C=O}$	$\nu_{C=C}$	NO_3^-	ClO_4^-
I	$CuL_{py}ClO_4 \cdot H_2O$		1590s	1540vs	1525vs		1100vs,br, 625m
II	$CuL_{py}NO_3 \cdot H_2O$		1590s	1545vs	1525vs	1380vs, 840w	
III	$CuL_{hm}ClO_4 \cdot H_2O$	3325s, 3265s	1595s	1545vs	1525vs		1100vs,br, 625m
IV	$CuL_{pupy}(ClO_4)_2 \cdot 0.5H_2O$		1635s		1595vs		1100vs,br, 625s

TABLE II. EPR Parameters of Cu–Complexes I–IV and Cu_2Zn_2superoxide Dismutase

		$g_\perp$	$g_\parallel$	$A_\parallel$		
				(G)	10^{-4} x (cm^{-1})	$\lvert g_\parallel/A_\parallel \rvert$
I	$CuL_{py}ClO_4 \cdot H_2O$	2.053	2.239	175	183	122
II	$CuL_{py}NO_3 \cdot H_2O$	2.049	2.242	178	186	120
III	$CuL_{hm}ClO_4 \cdot H_2O$	2.049	2.239	183	191	117
IV	$CuL_{pupy}(ClO_4)_2 \cdot 0.5H_2O$	2.040	2.226	160	166	134
	Cu_2Zn_2SOD [13]	2.087	2.268	134	142	160

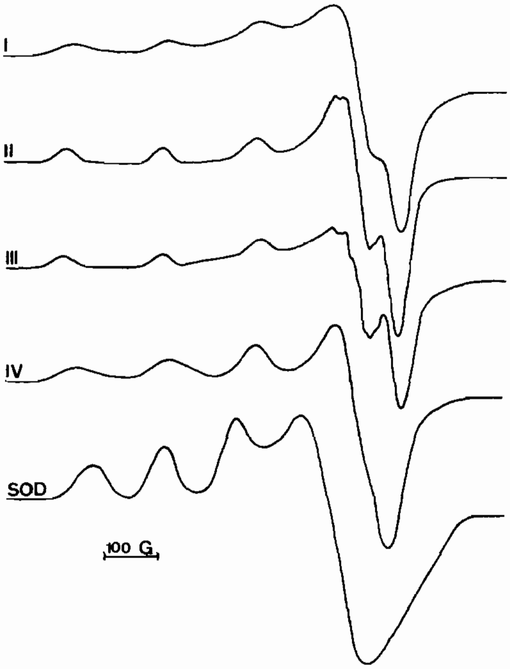


Fig. 4. X-band EPR spectra of Cu–complexes I–IV. I, $CuL_{py}(ClO_4) \cdot H_2O$; II, $CuL_{py}(NO_3) \cdot H_2O$; III, $CuL_{hm}(ClO_4) \cdot H_2O$; IV, $CuL_{pupy}(ClO_4)_2 \cdot 0.5H_2O$. Recording conditions: modulation amplitude 10 G, modulation frequency 100 kHz, microwave power 20 mW, microwave frequency 9.24 GHz, temperature 77 K.

Electronic Absorption

Comparative studies on the electronic absorption were useful in the 600–700 nm region. The broad absorption of the Cu–complexes I–III at 620 nm is typical for d–d transitions of Cu(II) in a weak tetragonal field. A marked red shift by 90 nm to 710 nm is seen when the di-Schiff-base IV is used. There is again a striking similarity to the copper binding site of Cu_2Zn_2superoxide dismutase, especially when the zinc binding site was empty [14]. In fact, the absorption maximum is exactly between those of the fully loaded Cu_2Zn_2superoxide dismutase and the Cu_2E_2superoxide dismutase. Furthermore, the absorption coefficient of the di-Schiff-base complex was similar to that of the enzyme bound copper (Table III). When IV was dissolved in methanol the absorption maximum remained at essentially the same wavelength region at 705 nm and the absorption coefficient rose to 150.

Superoxide Dismutase Activity

The inhibition of the reduction of nitroblue tetrazolium by superoxide dismutase and superoxide-dismutase-mimicking complexes were used [10]. The Cu–complexes I–III did not display considerable activity, although they were better superoxide dismutase mimicks compared to $Cu(tyr)_2$ and $Cu(lys)_2$. IV had the most distinct activity which came

TABLE III. Visible Electronic Absorption of Active Site Models and Cu_2Zn_2superoxide Dismutase

Copper complex	Solvent	nm	ε
I $CuL_{py}ClO_4 \cdot H_2O$	CH_3OH[a]	620	82
II $CuL_{py}NO_3 \cdot H_2O$	CH_3OH[a]	625	90
III $CuL_{hm}ClO_4 \cdot H_2O$	CH_3OH[a]	610	75
Cu_2Zn_2superoxide dismutase	H_2O	680	150
IV $CuL_{pupy}(ClO_4)_2 \cdot 0.5H_2O$	H_2O	710	101
Cu_2E_2superoxide dismutase	H_2O	740	140

[a] Insoluble in H_2O.

TABLE IV. Comparison of Superoxide Dismutase Activity of the Bovine Enzyme and Active Site Analogues. 0.5 ml of the Reaction Volume Contained 0.62 mM Nitro Blue Tetrazolium, 0.2% (*w/v*) Gelatine, 50 μM Xanthine, 0.18 μM Xanthine Oxidase, 20 mM HEPES Buffer, pH 7.4, 150 mM NaCl, Copper Complexes in Various Concentrations. A_{540} was Recorded at 23 °C

Copper complex	Concentration of chelated Cu(II) for 50% inhibition of formazane generation (μM)
$Cu(lys)_2$[a]	86
$Cu(tyr)_2$[a]	45
I $CuL_{py}ClO_4 \cdot H_2O$	29
II $CuL_{py}NO_3 \cdot H_2O$	24
III $CuL_{hm}ClO_4 \cdot H_2O$	13
IV $CuL_{pupy}(ClO_4)_2 \cdot 0.5H_2O$	1.4
Cu_2Zn_2SOD	0.04

[a]Data from ref. 10.

close to 3% of the native enzyme and was 60 times higher compared to $Cu(lys)_2$. In fact, the efficiency was not too far away from an iron superoxide dismutase (Table IV).

Conclusion

Acetate and biuret-type copper complexes were used in the past to mimick superoxide dismutase activity [15]. The copper ligands were oxygen and saturated nitrogen atoms bound to the Cu(II). However, these ligands are unsuitable for the transient Cu(I) coordination during the catalytic redox cycle.

In the native Cu_2Zn_2superoxide dismutase the copper is surrounded by four imidazolate nitrogens. Unlike common amino groups of aliphatic amino acids, these nitrogens are unsaturated and are at the same time very appropriate to bind Cu(I). The flexible situation in the polypeptide backbone allows the proper arrangement for both square planar or tetrahedral coordination around the respective oxidized copper species. Thus, the present aim was to simulate this flexibility employing . low molecular weight mononuclear copper chelator containing four unsaturated nitrogen atoms. The di-Schiff-base copper complex, being a tetradentate chelate, fulfilled these structural criteria quite well and proved to be a functional analogue of Cu_2Zn_2-superoxide dismutase.

Acknowledgements

This study was supported by DFG grant We 401/17-1 and in part by the Fonds der Chemischen Industrie. Thanks go to Mrs. Helga Heinemann for expert technical help.

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