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# A five-coordinate copper complex with superoxide dismutase mimetic activity from *Streptomyces antibioticus*

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**Summary.** A Cu(II) complex of desferrithiocin from *Streptomyces antibioticus* was prepared and characterized. The first shell atoms, including one nitrogen and four oxygens, were arranged around the copper in a square-planar pyramide. Due to the axially Jahn-Teller-distorted Cu-O distance at 224.7 pm, a distinct $Cu_2Zn_2$superoxide dismutase mimetic activity was measured. The Cu-complex survived 600 μM bovine serum albumin and the thermodynamic stability ($pK = 17.4$) was not very different from that of Cu-EDTA. The electronic absorption properties, circular dichroism and electron paramagnetism were in accordance with those of the type-II copper species.

**Key words:** Cu-desferrithiocin — Crystal structure — Superoxide dismutase activity — Stability

## Introduction

Superoxide dismutase catalyse the proton-dependent dismutation of superoxide into dioxygen and hydrogen peroxide (Gärtner and Weser 1986). Out of the three known enzymes, including manganese and iron proteins, $Cu_2Zn_2$superoxide dismutase is the catalytically most efficient species. In protein-free solution many low-$M_r$ copper complexes display identical or even higher rate constants compared to that of $Cu_2Zn_2$superoxide dismutase (Deuschle and Weser 1985). Unfortunately, these superoxide-dismutase-mimicking complexes are not equipped to survive physiological conditions.

Superoxide is generated in many biological systems in the course of electron-transfer reactions, autoxidizing molecules and redox-active enzymes. Superoxide is fairly reluctant in its reactions. In the presence of both superoxide and hydrogen peroxide and a reduced transition-metal ion, for example Fe(II) and Cu(I), the well-known metal-driven Haber-Weiss reaction takes place (Fenton and Jackson 1899; Haber and Weiss 1934; Bielski and Cabelli 1986). The hydroxyl radical is one of the most powerful oxidants and is thought to belong to the most reactive species. It has been assigned as being responsible for the breakdown of many biopolymers, including polynucleotides, carbohydrates and proteins (Hartmann et al. 1985). There is also frequently membrane damage and lipid peroxidation. As a consequence, there is considerable evidence to indicate the necessity of minimizing uncontrolled superoxide generation in biological systems. In extracellular fluids only negligible concentrations of superoxide dismutase are detectable. Particularly in blood plasma, where leucocytes and spurious metal ions are able to generate free radicals during inflammatory processes, scavenging of superoxide both catalytically and stoichiometrically should provide an increased protection against oxygen-free radicals.

A novel siderophore called ferrithiocin was isolated from *Streptomyces antibioticus* (Naegeli and Zaehner 1980). Apart from other transition metals, it has a fairly high specificity for the efficient chelation of iron (Peter 1985). Due to the prominent biochemical role of copper, we were interested in its reactions with this new apo-siderophore. Desferrithiocin was titrated with Cu(II) salts in an attempt to crystallize a defined complex suitable for X-ray diffraction studies. The structural work was paralleled by electronic absorption, magnetic measurements and circular dichroism. Special emphasis was placed on its

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possible $Cu_2Zn_2$superoxide dismutase activity and its ability to survive biological fluids.

## Materials and methods

*Materials.* Desferrithiocin was kindly donated from Ciba-Geigy, Basel (Peter 1985). Xanthine and nitroblue tetrazolium chloride were from Serva, Heidelberg, and xanthine oxidase from Boehringer, Mannheim. $Cu_2Zn_2$superoxide dismutase was prepared from bovine erythrocytes (Gärtner et al. 1984). The 1:1 copper complex of desferrithiocin was obtained by titration of 10 mM aqueous solutions (10 ml) of each of $CuSO_4$ and desferrithiocin. A green complex crystallized successfully at 4° C. It was washed twice with ice-cold water and dried in the presence of air.

*Spectrometry.* Electronic absorption was measured on a Hitachi U-2000 spectrophotometer. Electron paramagnetic resonance (EPR) spectroscopy was carried out on a Varian E 109 at modulation amplitude 10 G (1 mT), modulation frequency 100 kHz, microwave power 10 mW, microwave frequency 9.24 GHz, temperature 100 K. Circular dichroism was measured on a Jasco J-20 spectropolarimeter. Copper was quantified on a Perkin-Elmer atomic absorption unit equipped with a graphite furnace.

*Crystal structure determination.* A needle-shaped crystal of the green-coloured compound was mounted on a glass fiber. All X-ray investigations were performed on the diffractometer CAD4 (Enraf-Nonius, Delft) with graphite monochromated CuK$\alpha$ radiation. The compound crystallizes in the orthorhombic space group $P2_12_12_1$ with the lattice parameters $a=698.2(3)$ pm, $b=1108.5(3)$ pm, $c=166.9(4)$ pm and $Z=4$. In the $\theta=3$–65° range 1309 intensity data were collected with the $\omega/\theta$ scan. After absorption correction and averaging, 1129 unique reflections remained with an intensity $I>3.\sigma(I)$. The structure was solved by Patterson methods and subsequent difference Fourier synthesis, which also revealed the positions of the H atom. The absolute configuration was derived from the previous known enantiomer. All atoms (non-H) were assigned anisotropic thermal parameters, the H positions fixed the isotropic temperature factors. The final refinement converged to $R=0.033$. All calculations were performed on a DEC Micro VAXII with the program package VAXSDP. The positional and thermal parameters and further details of the crystal structure determination have been deposited as Supplementary Publication No CSD-53208. Copies may be obtained through the Fachinformationszentrum Energie, Physik, Mathematik GmbH (D-7514 Eggenstein-Leopoldshafen 2).

*Superoxide dismutase assay.* Superoxide dismutase activity was measured using the nitroblue tetrazolium assay (Younes and Weser 1976). One millilitre of the reaction volume contained 620 µM nitroblue tetrazolium chloride, 0.25% gelatin, 50 µM xanthine, 0.18 µM xanthine oxidase in 80 mM Hepes buffer, pH 7.6. Copper complexes in various concentrations were added to determine the Cu concentration required to suppress the formation of formazan by 50% ($ID_{50}$). Absorption measurements were carried out in a 1-cm light-path cell at 540 nm. The temperature was 25° C. In order to ascertain whether or not the xanthine oxidase used in the above test is inhibited by the copper complex employed, the xanthine oxidase activity was examined separately. Prior to use, the enzyme was freed from EDTA by gel filtration over Sephadex G-50 in the same buffer. The copper complex was added at the former defined $ID_{50}$ concentration. Uric acid formation was determined at 293 nm.

## Results and discussion

### *Electronic absorption*

Desferrithiocin reacted with copper successfully and a green complex was obtained. In order to determine the metal/ligand ratio, an aqueous solution of desferrithiocin was titrated with $CuSO_4$. Electronic absorption between 500 and 250 nm was used to measure the stoichiometry of the resulting copper complex. In the difference spectrum both the linear increase at 348 nm and 250 nm and the decline at 306 nm and 398 nm continued until equimolar concentrations of copper and desferrithiocin were reached (Fig. 1). In contrast to the other known metal complexes of 1:2 stoichiometry, including iron, cadmium and zinc, Cu-desferrithiocin seems to form the 1:1 complex preferentially.

The ultraviolet absorption of the Cu-desferrithiocin is red-shifted compared to the free ligand with strong absorption bands at 348 nm ($\varepsilon_{Cu}=9500\ M^{-1}\ cm^{-1}$) and at 400 nm ($\varepsilon_{Cu}=2000\ M^{-1}\ cm^{-1}$) (Fig. 2). There is an interesting similarity between these absorption bands and those of haemocyanin (347 nm, $\varepsilon_{Cu}=9000\ M^{-1}\ cm^{-1}$; 700 nm, $\varepsilon_{Cu}=500\ M^{-1}\ cm^{-1}$) (van Holde 1967). The electronic absorption near 348 nm can be assigned to a charge transfer between Cu(II) and oxygen. Unlike the electronic absorption of desferrithiocin in the visible region, Cu-desferrithiocin absorbs near 720 nm ($\varepsilon_{Cu}=100\ M^{-1}\ cm^{-1}$). This absorption is similar to that of nitrogen- and oxygen-coordinated cooper found in many other

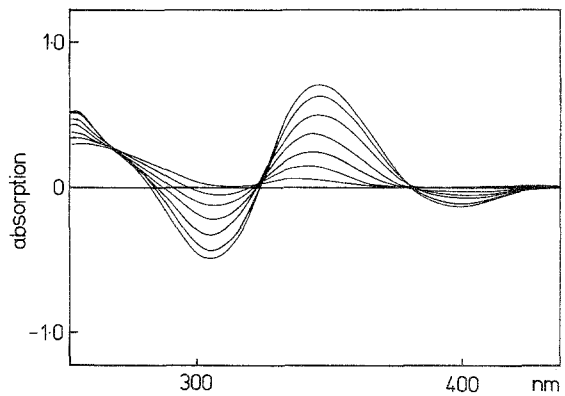


**Fig. 1.** Titration of desferrithiocin with $CuSO_4$. Difference spectrum of a 100 µM aqueous desferrithiocin solution. $CuSO_4$ (2.5 mM) was added in eight steps

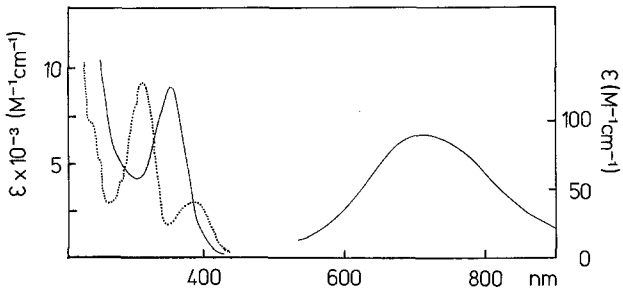


**Fig. 2.** Electronic absorption of aqueous desferrithiocin (· · · · ·) and Cu-desferrithiocin (——)

complexes, for example Cu-EDTA and the type II copper (Reinhammar and Malmström 1981).

*Circular dichroism*

The circular dichroism of Cu-desferrithiocin is rather complex (Figs. 3, 4). There are positive Cotton extrema at 600 nm, 380 nm and 265 nm, and negative Cotton bands above 700 nm, a double band at 340 nm and 310 nm and one at 235 nm. Desferrithiocin alone is also optically active and has weak Cotton bands at 380 nm (+), 330 nm (+), 290 nm (−) and 240 nm (+).

The characteristic chiroptical properties of Cu-desferrithiocin are convenient for the assignment of the stability of this complex in the presence of competing, biologically occurring, copper-binding ligands. Addition of equimolar bovine serum albumin does not change the spectrum

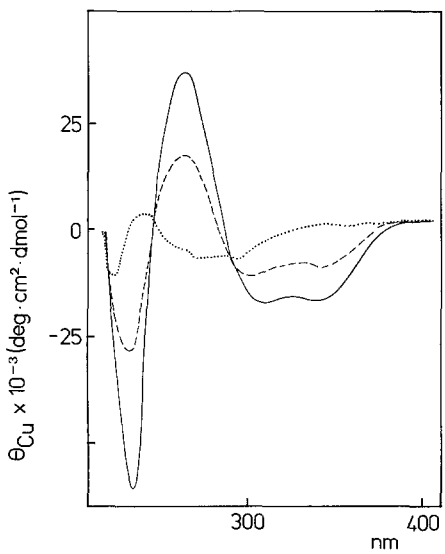


**Fig. 3.** Circular dichroism of desferrithiocin (· · · · ·), Cu-desferrithiocin (——) and Cu-(desferrithiocin)$_2$ (- - - -) in water

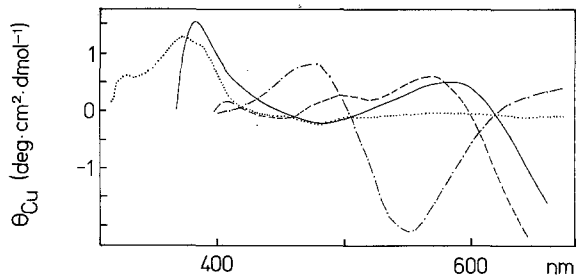


**Fig. 4.** Circular dichroism of Cu bovine serum albumin (- · - · -), desferrithiocin (· · · · ·), Cu serum albumin + desferrithiocin (- - - -) and Cu-desferrithiocin (——). The albumin concentration was 600 μM each, dissolved in 40 mM Tris/HCl at pH 8.0

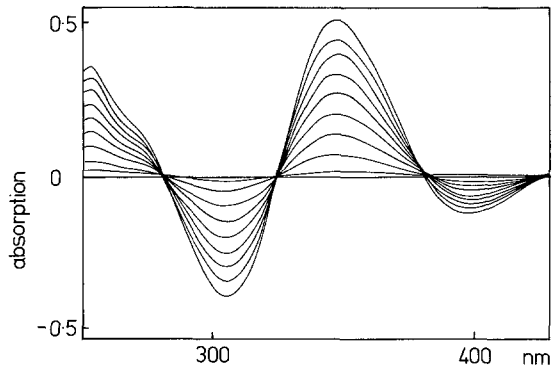


**Fig. 5.** Titration of Cu-desferrithiocin with EDTA. Cu-desferrithiocin (100 μM in water) was titrated with EDTA in final concentrations of 20, 40, 60, 80, 100 and 1000 μM. The difference in the absorption at 348 nm was taken to determine the resulting concentration of Cu-desferrithiocin

significantly. Only a minor shift of the Cotton bands is noticed (Fig. 4).

*Thermodynamic stability*

The stability constant of Cu-desferrithiocin was quantified by the stepwise addition of EDTA to a solution containing copper(II) and desferrithiocin. The portion of the remaining Cu-desferrithiocin spectra was measured by monitoring the electronic absorption at 348 nm (Irving and Mellor 1955; Linss and Weser 1987). At this wavelength the electronic absorption properties of Cu-EDTA are not perturbing. After the addition of an equimolar concentration of EDTA, 16% of the original electronic absorption of Cu-desferrithiocin was detectable (Fig. 5). The stability constant, $K$, of Cu-desferrithiocin can be calculated using the following equation:

$$\frac{K_{\text{Cu-desferrithiocin}}}{K_{\text{Cu-EDTA}}} = \frac{[\text{Cu-desferrithiocin}][\text{EDTA}]}{[\text{Cu-EDTA}][\text{desferrithiocin}]} = \frac{16.16}{84.84}$$

Assuming $K_{\text{Cu-EDTA}} = 6.3 \times 10^{18}$ (Peter 1985) the stability of Cu-desferrithiocin can be calculated to be $pK = 17.4$, i.e. not very different from that of the former complex.

*Electron paramagnetic resonance*

The EPR spectrum of Cu-desferrithiocin in water is similar to that of $CuSO_4$ (Fig. 6). When the spectrum is recorded in 50 mM Hepes buffer, pH 7.8, different spectra and hyperfine splittings at 1:2 and 1:1 stoichiometry of Cu-desferrithiocin were seen with $A_{II}$ values of 12.5 mT and 16.5 mT (125 G and 165 G), respectively.

*Crystal structure*

The structure of crystalline copper-desferrithiocin was elucidated by X-ray diffraction (Fig. 7). The first shell atoms around the copper were arranged in a square planar pyramid. The metal ion is bound to the oxygen of a phenolic hydroxyl group, one nitrogen of the thiazolin, an oxygen of the carboxyl group and two oxygen atoms originating from two $H_2O$. The axially bound water-oxygen O-2 exhibits a pronounced Jahn-Teller-distorted Cu-O distance of 224.7 pm with the greatest kinetic lability. O-1 belongs to a second water which is very tenaciously bound to the copper. In fact, it has the shortest bond length (192.2 pm) compared to all other oxygen atoms and almost reaches the Cu-N atomic distance of the unsaturated nitrogen N-1. The pronounced degree of covalency explains the survival of this copper complex in the presence of serum albumin. The O-1 oxygen remains firmly bound and does not interfere with the many peptide bonds. The axially coordinated ligand is kinetically too unstable to form a permanent coordination to serum albumin. Thus, this labile coordination site would be most convenient for catalysing superoxide dismutation in a way similar to $Cu_2Zn_2$superoxide dismutase.

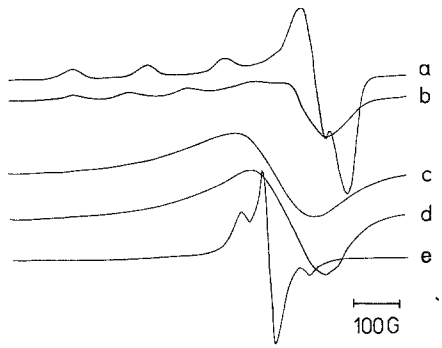


**Fig. 6.** EPR spectroscopy of Cu-desferrithiocin. EPR spectra were recorded in water, 40 mM Hepes buffer, pH 7.8 or in solid form. Instrumental conditions: microwave frequency 9.25 GHz, microwave power 20 mW, modulation amplitude 1mT and temperature 100 K. *a* Cu-desferrithiocin in Hepes buffer; *b* Cu-(desferrithiocin)$_2$ in Hepes buffer; *c* Cu-desferrithiocin in water; *d* Cu-(desferrithiocin)$_2$ in water; *e* Cu-desferrithiocin crystalline

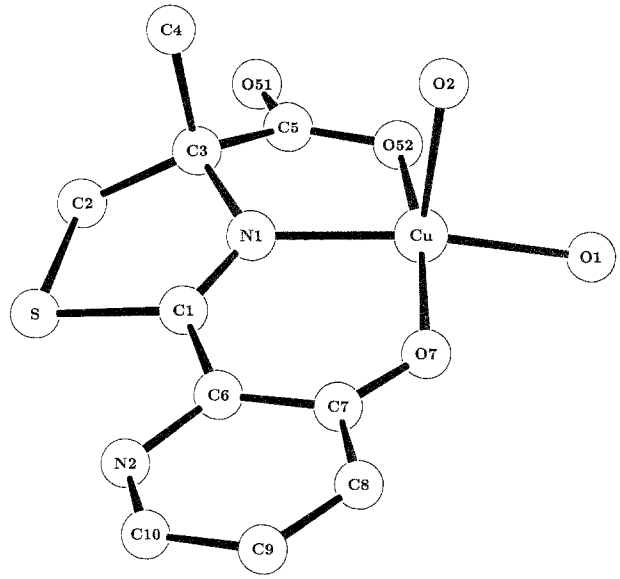


**Fig. 7.** Crystal structure of Cu-desferrithiocin

Another important condition for an active-centre analogue for $Cu_2Zn_2$superoxide dismutase is the flexible nature of the copper centre (Linss and Weser 1986, 1987). Both oxidation states of Cu(I) and Cu(II) are stabilized. The structure of the present complex can be regarded as lying between the copper complexes of the acetate and of the di-Schiff-base types described earlier (Gärtner and Weser 1986).

*Superoxide dismutase activity*

In the light of the structural data and the pronounced thermodynamic stability, it was attractive to search for a possible $Cu_2Zn_2$superoxide dismutase activity. The enzymic activity was determined by measuring the inhibition of the reduction of nitroblue tetrazolium. Cu-desferrithiocin displayed an activity of 2% to that of the native enzyme and was almost 50-times higher compared to Cu(Lys)$_2$ (Linss and Weser 1987). In contrast to most other complexes with superoxide-

**Table 1.** Bond length and angles of first shell atoms around the copper in Cu(II)-desferrithiocin

| Bond | Length (pm) | Bond angle | Size (deg.) |
|---|---|---|---|
| Cu-N1 | 190.2(4) | O1-Cu-O2 | 96.3(2) |
| Cu-O1 | 192.2(5) | O1-Cu-O7 | 91.5(2) |
| Cu-O7 | 193.9(4) | O1-Cu-O52 | 91.3(2) |
| Cu-O52 | 198.4(4) | O1-Cu-N1 | 166.5(3) |
| Cu-O2 | 224.7(5) | O2-Cu-O7 | 92.4(2) |
| | | O2-Cu-O52 | 101.0(2) |
| | | O2-Cu-N1 | 96.5(2) |
| | | O7-Cu-O52 | 165.9(2) |
| | | O7-Cu-N1 | 92.0(2) |
| | | O52-Cu-N1 | 82.3(2) |

Numbers in *parentheses* are standard deviations in the least-significant digits

**Table 2.** Comparison of superoxide dismutase activity of bovine $Cu_2Zn_2$superoxide dismutase and low-$m_r$ copper complexes

| Copper complex | Concentration of chelated Cu(II) for 50% inhibition of formazan formation (μM) |
|---|---|
| $Cu(Lys)_2$ | 86[a] |
| $Cu(Tyr)_2$ | 45[a] |
| Cu(py) | 29[a] |
| Cu(pupy) | 1.4[a] |
| Cu-desferrithiocin | 2.0 |
| $Cu_2Zn_2SOD$ | 0.04 |

Activity was determined using the tetrazolium blue assay. 1 ml of the reaction volume contained 620 μM nitro-blue tetrazolium chloride, 0.25% gelatin or 600 μM bovine serum albumin, 50 μM xanthine, 0.18 μM xanthine oxidase in 80 mM Hepes buffer, pH 7.6. Electronic absorption at 540 nm was measured at 25° C

[a] Data from Linss and Weser (1986)

dismutase-mimicking activity, this complex is active also in the presence of 600 μM albumin, where 11 μM Cu(II) was required to yield 50% inhibition of formazan formation. It should be kept in mind that this naturally occurring siderophore promises to be less toxic compared to the complexes of the di-Schiff-base type, which is important for using Cu-desferrithiocin as a functional analogue of superoxide dismutase in biological systems.

## Conclusion

Desferrithiocin has been shown to be a naturally occurring chelator. Apart from its strong iron-chelating capacity, Cu complexes with remarkable thermodynamic stability were formed. The 1:1 Cu complex survives competing biological chelators including serum albumin. The five-coordinate complex is able to allow reversible oxidation-reduction reactions of the copper without falling apart. A significant $Cu_2Zn_2$superoxide dismutase mimetic activity was noticed. What makes this Cu complex so intriguing is the fact that either $Cu_2Zn_2$superoxide dismutase or Cu-desferrithiocin is formed in biological systems.

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