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Superoxide dismutase mimetic activity of a cyclic tetrameric Schiff base *N*-coordinated Cu(II) complex

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A pulse radiolytic study using the cyclic tetrameric Schiff base *N*-coordinated copper complex Cu(TAAB)$^{2+}$ has been performed. The reaction of the Cu(TAAB)$^{2+}$ complex with superoxide revealed pseudo first-order characteristics with the rate constant of $k_2 = (2.9 \pm 0.5) \times 10^8$ mol^{-1} s^{-1} dm^3. The complex survived the presence of competing serum albumin in physiological concentrations. The complex stability constant $K = 1.15 \times 10^{18}$ (log $K = 18.06$) is two orders of magnitude higher than that of Cu(II)–serum albumin (log $K = 16.2$). Transient changes of the stability during the oxidation/reduction process and in the presence of 600 μmol l^{-1} albumin did not affect significantly either the electronic absorption of the complex or its catalytic activity.

Keywords: complex stability constant, cyclic tetrameric Schiff base, pulse radiolysis, SOD mimetic copper complex

Introduction

Cu_2Zn_2 superoxide dismutase (SOD) catalyses the proton dependent dismutation of superoxide into dioxygen and hydrogen peroxide (McCord & Fridovich 1969). At the catalytic cycle of SOD, the copper atom is reduced and oxidized consecutively (Fielden *et al.* 1974, Gartner & Weser 1986).

Copper is exclusively bound to unsaturated nitrogen in a distorted square planar arrangement in the active site of the enzyme. Upon reduction the copper atom remains positioned in a transient tetrahedral complex. Many low M_r copper complexes mimick SOD activity in aqueous systems. However, only a very limited number of thermodynamically stable copper complexes can survive competitive biological chelators. A suitable copper complex which would fulfil these requirements was thought to be the cyclic tetrameric Schiff base Cu(II) complex of tetrabenzo [*b*, *f*, *j*, *n*][1,3,9,13]tetraazacyclohexadecine (tetraanhydro-amino benzaldehyde), Cu(TAAB)$^{2+}$ (Figure 1).

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The structure and electrochemistry of this complex has been successfully elucidated (Yatsimirskij & Labuda 1980, Labuda *et al.* 1984, 1988, Labuda & Sima 1986, Labuda & Yatsimirskij 1991, Ďuračková *et al.* 1993).

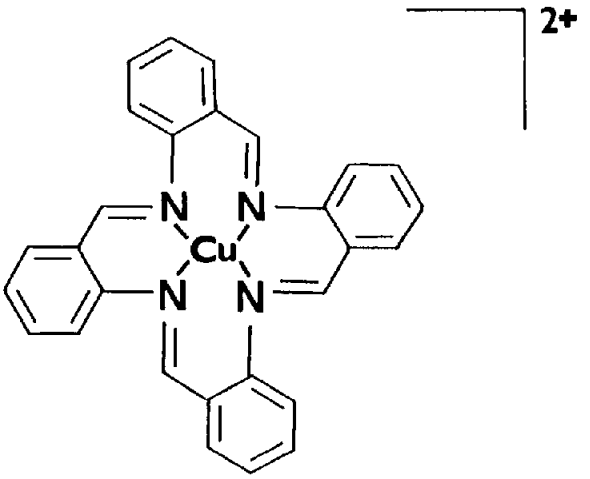

Figure 1. Macrocyclic complex Cu(TAAB)$^{2+}$.

Although the coordination geometry of Cu(II) in the complex and in the active center of SOD is different, four soft nitrogen donor atoms are likewise involved in the copper binding site. The planar system is surrounded by four phenyl ring systems which are connected in a flexible manner to the rigid macro-ring system. The outer hydrophobicity would ascertain the solubility in lipid layers of membranes or between the stacked base pairs of DNA or other polynucleotides. It was of interest to study the ability to survive physiological concentrations of serum albumin by monitoring the dichroic properties of a Cu(II)–bovine serum albumin (BSA) complex which may be formed from $Cu(TAAB)^{2+}$ via ligand substitution. On the functional side, pulse radiolysis was performed to obtain rate constants for the reaction of the complex with superoxide in a controlled aqueous system. In a separate study, the SOD mimetic activity was examined in the presence of physiological concentrations of serum albumin employing the xanthine–xanthine oxidase (X–XO) assay as well as the photoinduction of the riboflavine methionine system for superoxide production (Beauchamp & Fridovich 1971).

Materials and methods

BSA and HEPES were from Serva (Heidelberg, Germany); ascorbic acid, riboflavin and potassium ferricyanide were from Merck (Darmstadt, Germany); DL-methionine was from Calbiochem-Novabiochem (Bad Soden, Germany); X was from Koch Light (Colnbrook, Berks, UK); XO and catalase (CA) were from Boehringer Mannheim (Mannheim, Germany); 2990 U mg^{-1} SOD was from Sigma (St Louis, MO); human serum albumin (HSA) was from IMUNA (Šarišské Michal'any, Slovakia). 3-(4-Iodophenyl)-2-(nitrophenyl)-5-phenyltetrazolium chloride (INT) and other chemicals used were purchased from Lachema (Brno, Czech Republic).

$[Cu(TAAB)](NO_3)_2$ was synthesized as previously described (Melson & Busch 1964). Its chloride analog, $[Cu(TAAB)]Cl_2$, was prepared using anion exchange chromatography. Circular dichroism was recorded on a Jasco J 20A spectropolarimeter (Tokyo, Japan). Electronic absorption spectra were run on a Beckman spectrophotometer model DU 700 (München, Germany).

Pulse radiolysis experiments were carried out on a Febetron 705 accelerator. The optical detection system was composed of an Osram xenon lamp XBO 450 W4, a Schoeffel monochromator and an EMI 9659 photomultiplier unit. The signals were recorded on a Hewlett-Packard HP 5182 waveform recorder/ generator and subsequently stored and analyzed on a HP-9000/300 computer. All experiments were performed in quartz-distilled and pyrolyzed water. An aerated aqueous solution of KSCN (10 mmol l^{-1}) was used for monitoring the dose delivered per pulse assuming $G_e = 21522$ cm^{-1} mol^{-1} dm^3 per 100 eV at 500 nm for the transient $(SCN)_2^-$ species (Fielden 1982). Superoxide radicals were produced by a 100 ns radiation pulse of 1.8 MeV electrons in oxygen-saturated aqueous solutions and in the presence of 0.1 mol l^{-1} formate. Consequently, the G value of the superoxide rose to 60 (the yield of $O_2^{\cdot-}$ molecules per 100 eV of absorbed energy). Superoxide radical concentrations were in the range from 4.4×10^{-6} to 3.2×10^{-5} mol l^{-1} as determined from the dose of the radiation pulse and the UV absorption of the radical.

SOD assay

SOD activity was assayed by two methods.

(i) The X–XO system for the production of superoxide and INT for the superoxide detection (Durackova *et al.* 1993). The reaction mixture had a volume of 1.2 ml and contained: 42 μmol l^{-1} X, 82 μmol l^{-1} INT, with or without 0.6 mmol l^{-1} HSA and without or with variable concentrations of $Cu(TAAB)^{2+}$ in 1 mmol l^{-1} phosphate buffer, pH 7.8. The reaction was started by the addition of XO in a concentration to yield an absorbance change between 0.03 and 0.04 A min^{-1} at 510 nm and 25 °C omitting the complex.

(ii) The aerobic riboflavin-photosensitized oxidation of methionine (Beauchamp & Fridovich 1971) for the production of superoxide and INT. The reaction mixture (1.5 ml) contained: 21 μmol l^{-1} riboflavin, 10 mmol l^{-1} methionine, 0.21 mmol l^{-1} INT, with or without 0.6 mmol l^{-1} HSA and without or with variable concentrations of $Cu(TAAB)^{2+}$ in 1 mmol l^{-1} phosphate buffer, pH 7.8. The reaction was started by irradiation with a tungsten 30 W, 6 V light source (Narva) at a distance of 10 cm. The absorbance at 510 nm was measured after 15 min, when the production of superoxide reached a maximum. The absorbance was measured using an Opton PM2DL type spectro-photometer.

SOD mimetic activity of $Cu(TAAB)^{2+}$ is defined as the concentration of the complex required for 50% inhibition of the INT reduction at 510 nm by superoxide (IC_{50} value) or as $-\log IC_{50}$.

Results and discussion

The ability of $Cu(TAAB)^{2+}$ to catalyze the dismutation of superoxide anion radicals was examined using pulse radiolytically generated $O_2^{\cdot-}$. The major portion of superoxide decayed in the presence of $Cu(TAAB)^{2+}$ within 10 ms (Figure 2).

Analysis of the decay kinetics revealed a pseudo first-order reaction. The second-order rate constant was obtained by dividing the pseudo first-order rate constants over the respective $Cu(TAAB)^{2+}$ concentrations and has been calculated to be $(2.9 \pm 0.5) \times 10^8$ mol^{-1} s^{-1} dm^3. This rate constant is very similar to those observed for other Cu(II) chelates (Younes *et al.* 1978, Lengfelder *et al.* 1979, Felix *et al.* 1993).

The stability constant of $Cu(TAAB)^{2+}$ was evaluated to shed some light on the competitive Cu(II) binding capacity of serum albumin. Fortunately, no chiroptic properties of the active site analog perturbed the 559 nm region (Figure 3).

The characteristic negative Cotton band at 559 nm of the Cu–serum albumin adduct was used to follow

the displacement of Cu(II) from the low M_r chelate $Cu(TAAB)^{2+}$. The Cu(II)–BSA concentration was calculated from the obtained dichroic amplitude

$$y = 21887.4x \quad (1)$$

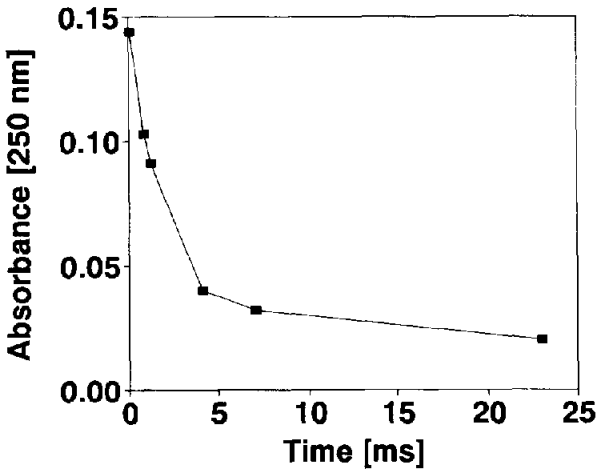

Figure 2. Decay of pulse radiolytically generated superoxide in the presence of $Cu(TAAB)^{2+}$. Electronic absorption at 250 nm. All measurements were carried out in oxygen-saturated solutions containing 2 μmol l^{-1} $Cu(TAAB)^{2+}$, 0.1 mol l^{-1} sodium formate, pH 7.2, $1111/T = 17.3\,°C$. Pulse length: 100 ns, dose: 60 Gy. For further details see Materials and methods. The second-order rate constant for the reaction of $Cu(TAAB)^{2+}$ (2 and 5 μmol l^{-1}) with superoxide (32 μmol l^{-1}) was obtained graphically using the method described by Felix *et al.* (1993).

where y is the dichroic amplitude (mdeg) and X is the concentration of Cu(II)–BSA (mol l^{-1}).

Both HSA and BSA have distinct Cu(II) binding sites on the N-terminal end (Shuff *et al.* 1992). The complexation ability of these binding sites is several orders of magnitude higher than that of many other unspecific binding sites in the protein. Thus, the binding site of serum albumin might be competing with the ligand TAAB for Cu(II) to form Cu(II)–BSA:

$$Cu(TAAB)^{2+} \leftrightarrow Cu^{2+} + TAAB \quad (2)$$

$$Cu^{2+} + BSA \leftrightarrow Cu(II)\text{–}BSA \quad (3)$$

$$Cu(TAAB)^{2+} + BSA \leftrightarrow Cu(II)\ BSA + TAAB \quad (4)$$

The stability constant of the $Cu(TAAB)^{2+}$ complex relative to that of Cu(II)–BSA can be calculated. From (2)–(4) it follows:

$$\beta_{rel.} = \frac{K_{Cu(II)\ BSA}}{K_{Cu(TAAB)^{2+}}} = \frac{[Cu(II)\text{–}BSA][TAAB]}{[BSA][Cu(TAAB)^{2+}]} \quad (5)$$

where the equilibrium concentrations are

$$[TAAB] = [Cu(II)\text{–}BSA]$$

$$[Cu(TAAB)^{2+}] = [Cu(TAAB)^{2+}]_0\ [Cu(II)\text{–}BSA]$$

$$[BSA] = [BSA]_0\text{–}[Cu(II)\text{–}BSA]$$

The concentration of Cu(II) BSA was calculated from (1). From (5) the absolute value of the stability constant

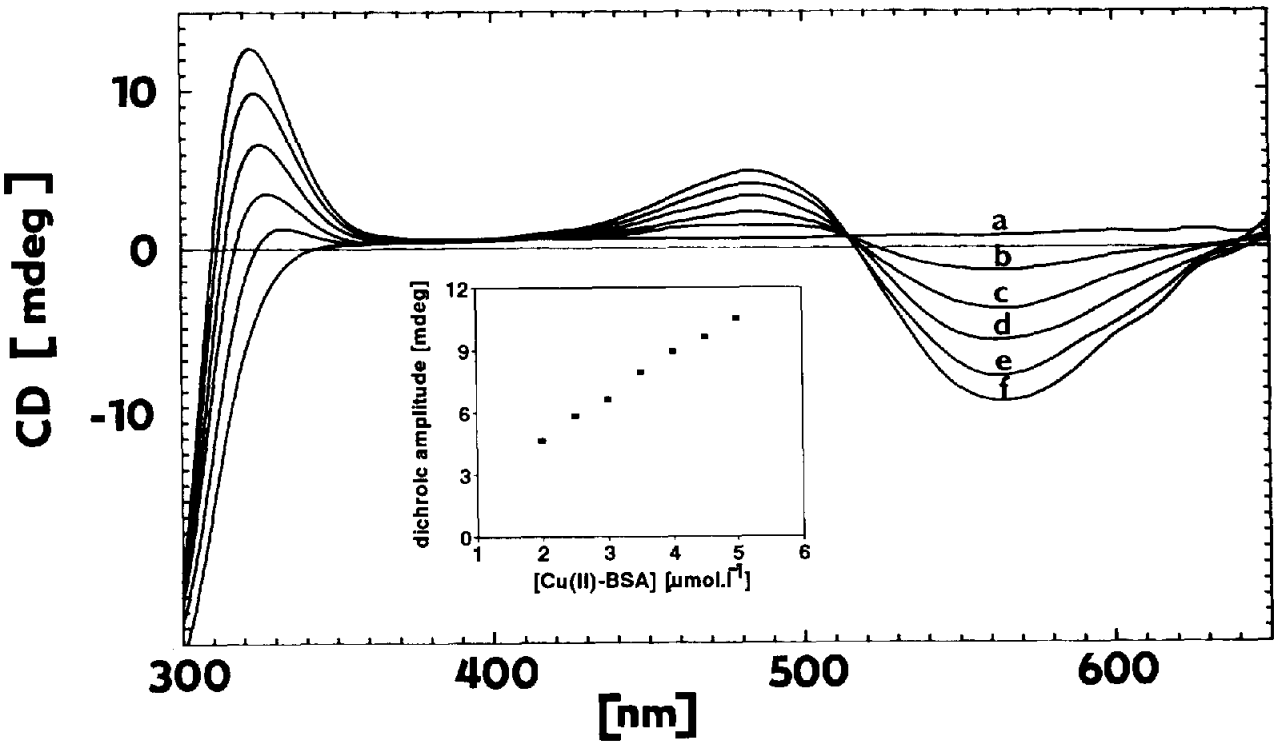

Figure 3. Circular dichroism of BSA titrated with rising concentrations of $CuSO_4$ in mmol l^{-1}: **a** (0), **b** (0.1), **c** (0.2), **d** (0.3), **e** (0.4), **f** (0.5). The serumalbumin concentration was 0.5 mmol l^{-1}. Inset: Calibration curve of dichroic amplitude at 559 nm versus the Cu(II) concentration.

Table 1. Determination of the $Cu(TAAB)^{2+}$ stability constant relative to Cu(II)–BSA

a (mdeg)	$[CuTAAB^{2+}]_0$	$[BSA]_0$	$[Cu(II)\text{–}BSA] \approx [TAAB]$	$[CuTAAB^{2+}]$	[BSA]	$\beta_{rel.}$
	(μmol l^{-1})	(μmol l^{-1})	(μmol l^{-1})	(μmol l^{-1})	(μmol l^{-1})	
0.129	50	500	5.88	44.12	494.12	0.002
0.217	100	500	9.92	90.08	490.08	0.002
0.529	150	500	24.10	125.90	475.90	0.010
0.819	250	500	37.35	212.65	462.65	0.014
1.139	350	500	51.94	298.06	448.06	0.020
1.554	450	500	70.85	379.15	429.15	0.029
1.292	500	500	58.93	441.07	441.07	0.018

All titrations were carried out in 5 mmol l^{-1} HEPES buffer (pH 7.0).
a, the dichroic amplitude of Cu(II)–BSA.

of the complex $Cu(TAAB)^{2+}$ can be calculated. The stability constant for Cu(II)–BSA was obtained from $\log K = 16.2$ (Linss & Weser 1987) and was 1.58×10^{16}. Assuming this value for Cu(II), the corresponding constants for $Cu(TAAB)^{2+}$ are $\log K = 18.06$ ($K = 1.15 \times 10^{18}$) and $\beta_{rel.} = 0.014 \pm 0.004$, respectively (Table 1). In conclusion, $Cu(TAAB)^{2+}$ is of extra-ordinary stability and well able to survive biological chelators.

The stability of $Cu(TAAB)^{2+}$ in the transient changes of the oxidation/reduction state during the dismutation reaction (Labuda *et al.* 1984) was also studied. $Cu(TAAB)^{2+}$ was completely reduced with a 2-fold molar excess of ascorbic acid to the blue $Cu(TAAB)^{+}$. After this reduction, the re-oxidation was accomplished by ferricyanide to yield the brown $Cu(TAAB)^{2+}$. The respective concentrations of the oxidized and reduced forms were calculated using the molar absorption coefficients: for $Cu(TAAB)^{+}$ = 5200 cm^{-1} mol^{-1} dm^3 at 660 nm and for $Cu(TAAB)^{2+}$ = 43000 cm^{-1} mol^{-1} dm^3 at 268 nm (Yatsimirskij & Labuda 1980). The calculated concen-trations were within the range of (43 ± 3) μmol l^{-1} which is comparable with the initial concentration of $Cu(TAAB)^{2+}$ (40 μmol l^{-1}).

Ferricyanide changes additively the spectra of the complex (Figure 4, curve 5). After the complex reduction and oxidation the original spectrum was observed (Figure 4, curve 4). Spectrum 4 is the additive spectrum 2 omitting ferricyanide plus the ferricyanide contribution of curve 1. The spectrum of the reduced form is different from that of the oxidized form (Figure 4, curve 3).

The effect of strongly chelating serum albumin at concentrations of 0.1 and 0.6 mmol l^{-1} on the SOD mimetic activity of the complex was further examined using the X–XO or riboflavin–methionine assays. $Cu(TAAB)^{2+}$ alone within the concentration range of 0.15–15 μmol l^{-1} did not affect the XO activity (the urate formation was not affected). The presence of 0.1 mmol l^{-1} albumin diminished the XO activity to 80%. At 0.6 mmol l^{-1} albumin the XO activity was of the order of 30% compared with the activity in the absence of albumin.

For $Cu(TAAB)^{2+}$ in the presence of 0.1 mmol l^{-1} albumin, an identical IC_{50} value in both systems, i.e.

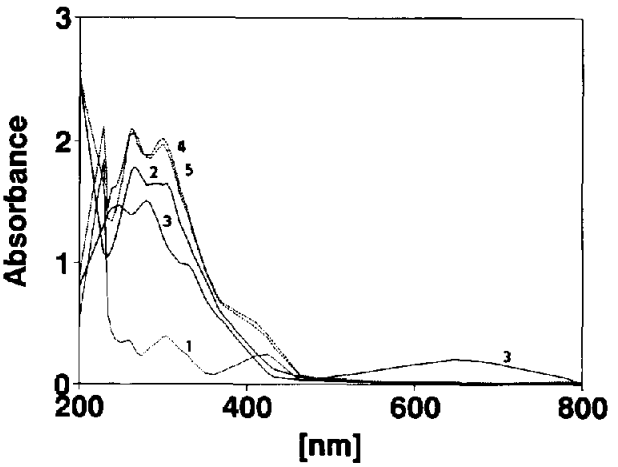

Figure 4. Electronic absorption spectra of ferricyanide (250 μmol l^{-1}) (**1**); $Cu(TAAB)^{2+}$ (40 μmol l^{-1}) (**2**); 40 μmol l^{-1} $Cu(TAAB)^{+}$ prepared by reduction with 40 μmol l^{-1} ascorbate (**3**); 40 μmol l^{-1} $Cu(TAAB)^{2+}$ previously reduced to $Cu(TAAB)^{+}$ and after 30 min reoxidized by 250 μmol l^{-1} of ferricyanide (**4**); 40 μmol l^{-1} of $Cu(TAAB)^{2+}$ plus 250 μmol l^{-1} ferricyanide (**5**).

X–XO and riboflavin–methionine, was observed. Due to a dramatic rise in viscosity at the higher concentration of albumin, the use of the X–XO assay was unsuccessful. The riboflavin–methionine system was employed instead. The activity of the complex was compared with that of SOD under the same conditions. A 4.8-fold higher concentration of $Cu(TAAB)^{2+}$ was essential to survive 100 μmol l^{-1} BSA in the X–XO assay. In the case of the riboflavin–methionine assay, the SOD activity survived the presence of even 600 μmol l^{-1} serum albumin.

Conclusion

Although the cyclic tetrameric Schiff base *n*-coordinated Cu(II) complex $Cu(TAAB)^{2+}$ differs from the known copper binding center in SOD and the analog CuPuPy (Felix *et al.* 1993), it represents a functional SOD model. It

was intriguing to realize the extraordinary stability of the $Cu(TAAB)^{2+}$ complex in the presence of competing biological chelators. Thus, $Cu(TAAB)^{2+}$ might be a valuable tool for controlling superoxide levels in hydrophobic media. Four phenyl residues are expected to substantially improve the solubility of this complex in membrane lipids and other non-aqueous systems.

Acknowledgments

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