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Pulse radiolytically determined superoxide dismutase mimicking activity of copper–putrescine-pyridine, a diSchiff base coordinated copper complex

Klaus Felix, Edmund Lengfelder, Dirk Deters* & Ulrich Weser*

*Strahlenbiologisches Institut der Ludwig-Maximilians-Universität München, München and *Anorganische Biochemie, Physiologisch-chemisches Institut der Eberhard-Karls-Universität Tübingen, Tübingen, Germany*

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In order to exclude possible interferences in the many indirect superoxide dismutase (SOD) activity measurements using the copper–putrescine-pyridine complex (Cu–PuPy) a pulse radiolytic study on this diSchiff base copper complex has been devised and sucessfully performed. The reaction kinetics and rate constants of pulse radiolytically generated superoxide in the presence of Cu–PuPy reveal pseudo first-order characteristics. The rate constant ($k_2 = 6 \pm 1 \times 10^8$ M^{-1} s^{-1}) is comparable to that of an Fe–SOD and is approximately a factor of 3 lower than that of bovine Cu_2Zn_2–SOD. The superoxide dismutating activity of Cu–PuPy shows a more pronounced temperature dependence compared with that of Cu_2Zn_2–SOD. Arrhenius analyses yielded activation energies of 7.8 ±0.3 and 4.6 ± 0.2 kcal mol^{-1} for Cu–PuPy and Cu_2Zn_2–SOD, respectively. The rate constant of the reaction of superoxide and Cu–PuPy is highest at pH 5.0. The possible application of Cu–PuPy for new therapeutic strategies on all types of inflammatory diseases appears to be promising.

Keywords: diSchiff base Cu–SOD mimetic copper complexes, hyperthermia, pulse radiolysis, reactive oxygen species (ROS)

Introduction

The pharmacological effects of clinically employed copper complexes are characterized by antiinflammatory, antiulcer, anticonvulsant, anticancer, antidiabetic and radioprotective activities. These activities are consistent with superoxide dismutase (SOD)-like activity and the role of copper complexes in the physiological response to these types of diseases (Sorenson 1982, Deuschle & Weser 1985). For the copper mediated catalytic dismutation of superoxide there is no difference whether or not the metal is bound to low molecular weight ligands or coordinated to amino acid residues within large protein molecules. As superoxide is a radical species, the copper driven reaction does not require any activation energy and the protein portion does not contribute to the observed reactivity. The protein moiety of the SOD seems to function as an electrostatic promotor of $O_2^{\cdot-}$ binding attributable to the specific arrangements of charged residues funneling the radical into the active centre (Fisher *et al.* 1991, Desideri *et al.* 1992, Djinovic *et al.* 1992). Unlike the intact enzyme, Cu_2Zn_2–SOD, where the copper is surrounded by four imidazolate nitrogens, the majority of synthetic complexes belong to the acetate- or biuret-type copper complexes where the ligands are unsuitable for transient Cu(I) coordination during the catalytic redox cycle. Neither of these copper complexes survives the presence of serum albumin. By way of contrast, Cu–PuPy, a well characterized active site analog of Cu_2Zn_2–SOD, is stable in serum, and mimics both the structure and function of the catalytic center of the enzyme (Linss & Weser 1986). The ability of Cu–PuPy to exhibit SOD

Address for Correspondence: U. Weser, Anorganische Biochemie, Physiologisch-chemisches Institut der Eberhard-Karls-Universität Tübingen, Hoppe-Seyler-Straße 4, D-7400 Tübingen, Germany. Fax: 49-7071-296391.

activity has been repeatedly demonstrated using indirect methods (Linss & Weser 1986, 1987, Miesel & Weser 1988). The aim of this study is to measure the direct reaction between this diSchiff base coordination copper complex with pulse radiolytically generated superoxide in order to assign the type of the reaction, i.e. stoichiometrical or catalytical, the order and rate constants of these reactions, and their dependence on pH and temperature.

Materials and methods

Cu–PuPy [*N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine (*N,N',N'',N'''*)–Cu(II)–diperchlorate] was prepared according to a modified method of Linss & Weser (1986). First, 380 μl (4 mmol) pyridine-2-aldehyde and 740 mg $Cu(ClO_4)_2 \cdot 6\,H_2O$ (2 mmol) were dissolved under constant stirring in 10 ml H_2O. Immediately after the dropwise addition of 200 μl (2 mmol) 1,4-diaminobutane in 3 ml H_2O the pH value of the reaction solution was adjusted to pH 5.5 with 0.1 M perchloric acid. Stirring was continued until blue needles of the copper complex started to grow. The suspension was stored at 4 °C for 24 h. The crystals were collected and washed with cold water and ethanol. They were allowed to dry at 50 °C. Cu_2Zn_2–SOD from bovine erythrocytes, putrescine and pyridine-2-aldehyde were from Sigma (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 mM) was used for monitoring the dose delivered per pulse assuming $G_E = 21522\ dm^{-3}\ mol^{-1}\ cm^{-1}$ 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 M formate. Consequently, the G value of the superoxide rose to 6.1 (yield of $O_2^{\cdot -}$ molecules per 100 eV of adsorbed energy). Superoxide radical concentrations were in the order between 1.2×10^{-6} and 4.8×10^{-5} M as determined from the dose of the radiation pulse and the UV absorption of the radical. Electron paramagnetic resonance (EPR) spectra of Cu–PuPy at different pH values were recorded on a Bruker ESP 300 E in the X-band region. The following parameters were chosen: modulation amplitude 5 G, modulation frequency 100 kHz, microwave power 20 mW, microwave frequency 9.49 GHz and temperature 100 K. Then, 50% (v/v) aqueous ethyleneglycol solutions containing 1 mM Cu–PuPy for EPR measurements were titrated with 0.1 M perchloric acid to yield pH values between pH 2 and 8.

Results

The ability of Cu–PuPy 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–PuPy within 4 ms (Figure 1, inset).

Analysis of the decay kinetics revealed a pseudo first-order reaction. The second-order rate constants were obtained by dividing the pseudo first-order rate constants over the respective Cu–PuPy concentrations. In order to determine whether or not the acceleration of the dismutation by Cu–PuPy proceeded in a genuine catalytical manner and not stoichiometrically, the complex was exposed to multiple pulses (high excess of $O_2^{\cdot -}$). No change of the decay kinetics was seen. The dismutation rates were reasonably constant at concentrations above 0.5 μM Cu–PuPy ($k_2 = 6 \pm 1 \times 10^8\ M^{-1}\ s^{-1}$). Increased rate constants were determined at lower concentrations of Cu–PuPy (Figure 1).

The complex catalyzed superoxide dismutation at essentially the same rate as observed with many known Cu(II) chelates (Younes *et al.* 1978, Lengfelder *et al.* 1979). It has been emphasized that the ligands alone did not show this catalytic activity. The pH dependence of the Cu–PuPy catalyzed $O_2^{\cdot -}$ dismutation exhibits a maximum at pH 5.0, which is close to the pK of $HO_2^{\cdot}/O_2^{\cdot -}$ (Figure 2).

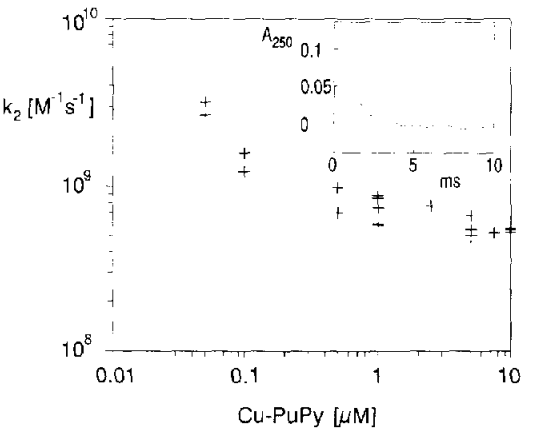

Figure 1. The second-order rate constant of the reaction between Cu–PuPy and superoxide at different concentrations of the copper complex. Superoxide: 19 μM, pH 7.2, T = 18 °C. Each point represents an average of three independent measurements. Inset: decay of pulse radiolytically generated superoxide in the presence of Cu–PuPy. Electronic absorption–time curve at 250 nm. The measurements were carried out in oxygen-saturated solutions containing 2 μM Cu–PuPy, 0.1 M sodium formate, pH 7.1, T = 22 °C. Pulse length: 100 ns, dose: 64 Gy. For further details see text.

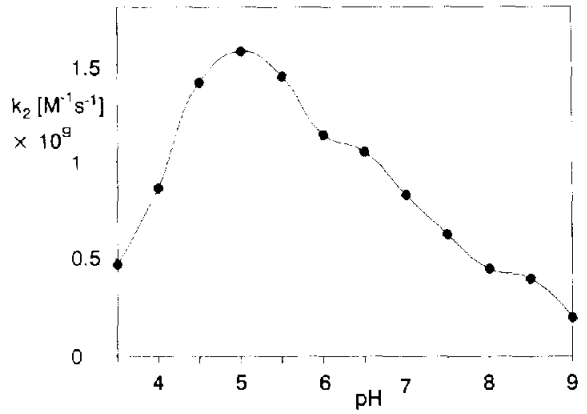

Figure 2. pH dependence of the rate constant of the reaction between superoxide and Cu–PuPy. Oxygen-saturated 0.1 M formate solutions containing 5 μM Cu–PuPy were used. Dose per pulse = 45 Gy. Data are expressed as mean values from four independent measurements. The standard deviation was less than 4%.

EPR measurements revealed no significant changes of the Cu–N coordination in the range between pH 3 and 8, but below pH 3 the imino nitrogens protonate and copper can dissociate from the chelator (Figure 3).

Increasing the temperature from 20 to 52 °C resulted in a remarkable activation of $O_2^{\cdot -}$ dismutation catalyzed by Cu–PuPy. Arrhenius analysis (Figure 4, inset) yielded activation energies of 7.8 ± 0.3 and 4.6 ± 0.2 kcal mol^{-1} for Cu–PuPy and Cu_2Zn_2–SOD, respectively. Thus, under physiological conditions (37.5 °C, pH 7.1) the second-order rate constant reaches a value of 1.1×10^9 M^{-1} s^{-1} (Figure 4), which is approximately a factor of 3 lower than that of the rate constant of Cu_2Zn_2–SOD from bovine erythrocytes under similar conditions (3.1×10^9 M^{-1} s^{-1}).

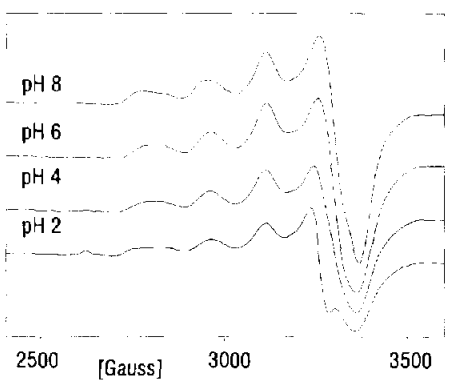

Figure 3. EPR spectra of Cu–PuPy at different pH values. The solutions contained 1 mM Cu–PuPy and 50% (v/v) ethyleneglycol (see Materials and methods).

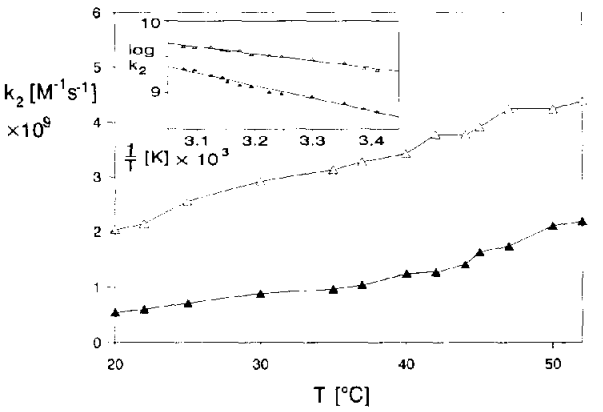

Figure 4. Temperature dependence of the rate constant k_2 for Cu–PuPy (▲) and Cu_2Zn_2–SOD (△). The inset shows the Arrhenius plots. For experimental details see the legend to Figure 1.

The rate of the spontaneous dismutation of superoxide at 52 °C was found to be about 4 times higher than that measured at 20 °C. Transient spectra recorded during the pulsing of oxygen-saturated 5 μM Cu–PuPy solution in the presence of 0.1 M formate are shown in Figure 5.

The observed maxima at 250 nm can be attributed to both Cu–PuPy and the overlapping electronic absorption of $O_2^{\cdot -}$. As with Cu–PuPy, a copper electronic transition near 410 nm is quite common for a number of type 1 and type 2 copper sites (Fee 1975, Ettinger & Kosman 1981, Gärtner & Weser 1986). Based on this copper complex and other copper–protein systems, possible contributors to this band include *d–d* transitions and unsaturated ligand–copper charge transfer (Solomon *et al.* 1976).

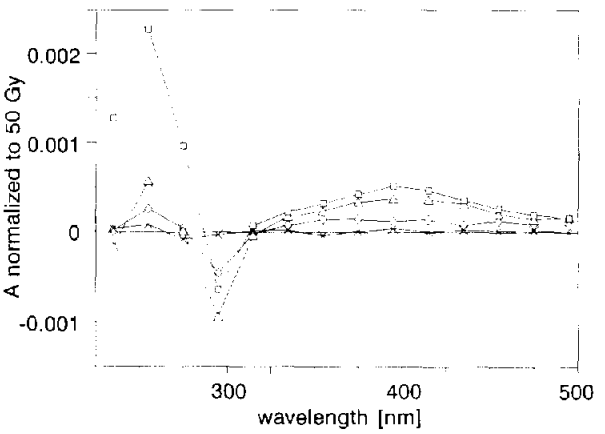

Figure 5. Transient electronic absorption spectra obtained on pulse radiolysis of aqueous solutions of 10 μM Cu–PuPy $O_2^{\cdot -}$ in 0.1 M formate, pH 7.0, doses per pulse 50 Gy. Spectra were recorded 200 μs (□), 1.9 ms (△), 7.2 ms (◇) and 23 ms (×) after the pulse.

The minimum at 295 nm originates from the electronic absorption of Cu(II)–PuPy. The progressive decline of these curves shows that after 23 ms all of the superoxide is converted into hydrogen peroxide and Cu–PuPy is completely reconverted into its Cu(II) oxidation state. The stability and reactivity of Cu–PuPy was also examined after the addition of EDTA, KCN and H_2O_2. The presence of EDTA, as a strong chelator, and cyanide, as a well known ligand and an extremely powerful inhibitor of the intact Cu_2Zn_2–SOD, was found to reduce the rate of dismutation of O_2^- considerably (Table 1).

With a 30-fold excess of hydrogen peroxide over Cu–PuPy the rate constant of the superoxide dismutation is progressively diminished.

Discussion

It was demonstrated that Cu–PuPy reacts with pulse radiolytically generated superoxide in a fast and efficient manner. In contrast to the indirect methods used in earlier studies to evaluate the SOD mimetic catalysis of Cu–PuPy, pulse radiolysis is a reliable method for the direct determination of both rate constants and reaction order. Furthermore, this method allows tracing of possible transient states of the copper complex generated in the course of the catalytical process.

At present we do not know the reason why the rate constant of Cu–PuPy is diminished compared with that of the intact cupric enzyme and the many known copper chelates of acetate or biuret origin. In the latter species the Jahn–Teller distorted axial ligand is exchangeable at a much faster rate compared with the O_2^- binding site of the diSchiff base complex. The magnetic coupling of the Cu(II) in the diacetate species should also be taken into consideration for the observed fast reaction with O_2^-. There are differences in the electronic saturation of either ring system when comparing the diSchiff base copper and the imidazolate copper of the intact enzyme. In the pyridine ligand the electronic situation is more delocalized compared with the facilitated electron transfer in the imidazolate ring systems. Unlike the Jahn–Teller distorted readily exchangeable axial H_2O ligands of the Cu(II) acetate species, both Cu–PuPy and Cu_2Zn_2–SOD are unable to exchange possible coordinated H_2O in this fast manner. For example, Cu_2 (indomethacin)$_4$ (Weser *et al.* 1980) has a rate constant almost 3 times higher than the k_2 of Cu_2Zn_2–SOD.

Table 1. Calculated second-order rate constants of the reactions between superoxide and Cu–PuPy in the presence of different inhibitors

Cu–PuPy (μM)	H_2O_2 (μM)	EDTA (μM)	KCN (μM)	Averaged rate constant k_2 ($M^{-1}\ s^{-1}$)
10			10	3.65×10^5
10		10		9.49×10^5
1		5		6.86×10^5
10	50			5.60×10^8
10	100			5.58×10^8
10	200			5.80×10^8
10	300			4.38×10^8
10	400			1.23×10^8

Conditions: 0.1 M Na-formate, pH 7.0, T = 20 °C. Mean values of the rate constant k_2 were determined at superoxide concentrations between 19 and 38 μM. Reproducibility was better than 4%.

The pulse radiolytic results show a marked increase of the reactivity of the superoxide radicals at elevated temperatures. The rising k_2 values during hyperthermic and slight acidic conditions may encourage the use of the Cu–PuPy complex as an efficient compound on a cellular level. For example, it is known that hyperthermia can produce regression of cancer in animal models and humans. The potential of hyperthermia in combination with radiation or antitumor drugs has become more evident from recent clinical investigations (Field 1989). The possibility that Cu–PuPy binds to DNA and DNA-associated proteins where it may act as a radiosensitizer ('chemical nuclease') by inducing strand scission cannot be excluded. In the presence of O_2, the primary radicals formed by ionizing radiation can produce O_2^- and peroxyl radicals which can be converted into additional superoxide. If Cu–PuPy is added, superoxide is used by the metal driven Haber–Weiss reaction to yield HO$^\cdot$ with the consequence of increased radiation damage. Recently, Chen & Sigman (1986) demonstrated that copper–phenanthroline produces strand scission in DNA and can serve as a useful tool in molecular biology, e.g. as a site-specific nuclease (Chen & Sigman 1987). Special attention should be given to possible SOD mimetic activity of Cu–PuPy in the presence of nitric oxide (NO), a vasodilator produced from arginine by NO synthase. Earlier studies (Gryglewski *et al.* 1986) showed that superoxide is involved in the breakdown of endothelium-derived vascular relaxing factor, which can be protected by the addition of SOD. Indirect studies strongly suggest a pronounced reactivity of SOD with NO (Murphy & Sies 1991). Saran *et al.* (1990) have pulse radiolytically measured the reaction of NO with superoxide, which proceeds at a rate of $k_2 = 5.6 \times 10^7\ M^{-1}\ s^{-1}$ at 37 °C. In the light of the

phenomenon that vascular tone is a factor in ischemia/reperfusion injury, Cu–PuPy could be an important protector for NO and potentiate vasodilatation by the elimination of superoxide which otherwise reacts to peroxonitrite ($ONOO^-$) and further to nitrate.

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