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The analogous reaction of diSchiff base coordinated copper and Cu_2Zn_2 superoxide dismutase with nitric oxide

Dirk Deters & Ulrich Weser

Anorganische Biochemie, Physiologisch-chemisches Institut der Eberhard-Karls-Universität Tübingen, Tübingen, Germany

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In addition to the well known catalytically accelerated O_2^- dismutation, Cu_2Zn_2 superoxide dismutase (SOD) reversibly reduces NO to NO^- with the consequence of a prolonged half-life of NO. This alternative reactivity was examined in the presence of the intact CuZn enzyme and a diSchiff base copper complex prepared from putrescine and pyridine-2-aldehyde (Cu–PuPy) which is known as a convenient active center analog of the former copper protein. The reaction of this SOD mimick with NO and NO^- was monitored by electronic absorption and electron paramagnetic resonance (EPR) spectroscopy via the formation of nitrosylmyoglobin. Cu–PuPy reacted up to three times faster with NO compared with Cu_2Zn_2 SOD and 15 times faster in comparison with $CuSO_4$ and copper EDTA. The oxidation rate of NO^- by Cu–PuPy was up to 300% higher compared with the reactivities of $CuSO_4$ and Cu EDTA. Cu_2Zn_2SOD reacted with NO^- to a neglible extent only. Catalytic characteristics could be observed in the course of the oxidation of NO^- in concentrations between 1 and 20 μM copper. Disturbances of the EPR properties suggested a modification of the chemical environment at the copper sites in both the copper complex and the enzyme. As a consequence, no further reactions of the nitrogen monoxides with the respective active centers were seen. In conclusion, Cu–PuPy appears to be an efficient moderator of the biochemical reactivity of nitrogen monoxides attributable to the observed increased half-life of NO.

Keywords: Cu–PuPy, Cu_2Zn_2 superoxide dismutase, diSchiff base Cu SOD mimetic copper complex, nitric oxide

Introduction

In 1987 it was suggested that NO may be identical with the endothelium-derived relaxing factor (EDRF) (Ignarro *et al.* 1987, Furchgott 1988). Prior to these reports NO was regarded as a highly toxic gas with no detectable biological importance (Snyder & Bredt 1992). Thus, it was intriguing to realize that macrophages are capable of releasing NO from L-arginine via a NO-synthase (Palmer *et al.* 1988) with the consequence that bacteria, fungi and tumor cells are destroyed (Keller 1973, Krahenbuhl & Remington 1974, Granger *et al.* 1980). In the process of blood vessel dilatation, NO is released after binding of acetylcholin to specific receptors on the endothelium. In the surrounding muscle layers, NO stimulates the formation of the second messenger cGMP which eventually causes the observed relaxation. NO is also involved in the processes of learning and memory, and is responsible for the damage of neurons after a stroke (Culotta & Koshland 1992).

Initially, the identification of NO as EDRF was obscured due to its pronounced reactivity with oxygen and water (1).

$$2NO + O_2 \rightarrow 2NO_2 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+ \quad (1)$$

In pulse radiolytic studies it was shown that NO can effectively react with superoxide radicals (Saran *et al.* 1990). During the reaction the formation of nitrate via transient peroxynitrite was noticed.

$$NO + O_2^- \rightleftarrows ONOO^- \rightarrow NO_3^- \quad (2)$$

Due to the scavenging of superoxide by superoxide dismutase (SOD), the reactivity of enzymically released NO from either L-arginine or pharmacological NO donors, including glyceryl trinitrate, is thought to be substantially prolonged (Moncada *et al.* 1986, Ignarro *et al.* 1987).

Address for correspondence: U. Weser, Anorganische Biochemie, Physiologisch-chemisches Institut der Eberhard-Karls-Universität Tübingen, Hoppe-Seyler-Straße 4, D-72076 Tübingen, Germany. Tel: (+49) 7071 29 63 91.

Reaction (2) is suppressed and NO can enter the above mentioned pathways. Furthermore, SOD protects NO in a different way against inactivation. [Cu(I)]–SOD reversibly reduces NO to yield the nitroxyl anion which does not react with $O_2^{\cdot-}$ (Murphy & Sies 1991).

$$NO \underset{[Cu(II)]-SOD}{\overset{[Cu(I)]-SOD}{\rightleftharpoons}} NO \qquad (3)$$

In most of the presently known SOD mimetic complexes, copper is coordinated in an acetate or biuret like manner where the ligands are unable to form stable complexes with transiently formed Cu(I) in the course of the observed redox cycle of the enzyme. The well characterized diSchiff base coordinated copper complex Cu–PuPy is an active site analog of Cu_2Zn_2SOD with pronounced SOD mimetic activities (Linss & Weser 1986, 1987, Miesel & Weser 1989, Felix *et al.* 1993). See Figure 1.

Attributable to the structural and functional similarities between the copper site of the intact enzyme and Cu–PuPy and the radical châracter of both superoxide anion and NO, it seemed promising to study the reactivity of the complex with NO and NO^-.

Either NO and its reduced form NO^- reacted with (met)myoglobin (MetMb; reaction 5; Kelm & Schrader 1988). This reaction was monitored using the electronic absorption of the myoglobin–NO adduct (nitrosylmyoglobin, MbNO; Figure 2).

$$Cu(I)–PuPy + NO \rightleftarrows Cu(II)–PuPy + NO^- \qquad (4)$$

$$NO^- + MetMb \rightarrow MbNO \leftarrow Mb - NO \qquad (5)$$

The rate of the reaction was evaluated by electronic absorption. The intactness of the chemical environment of the coordinated copper in Cu(II)–PuPy and Cu_2Zn_2SOD was monitored by electron paramagnetic resonance (EPR).

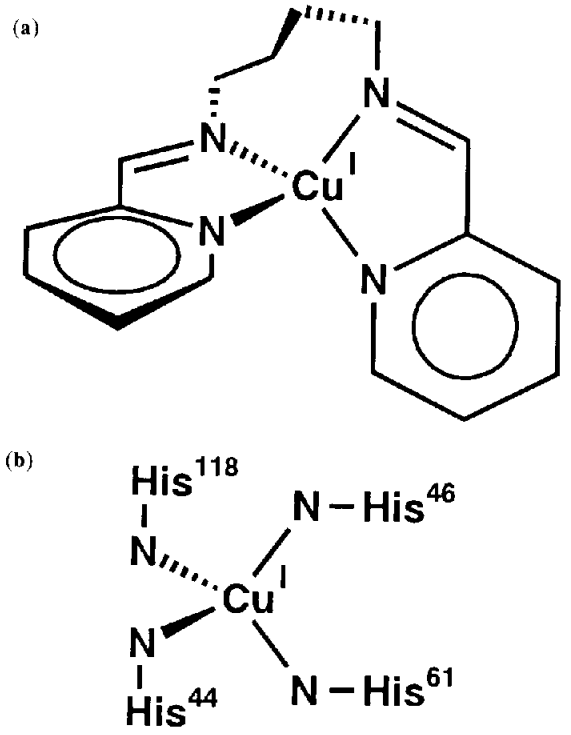

Figure 1. Cu–PuPy and Cu_2Zn_2SOD. (**a**) Structure of Cu(I)–PuPy. (**b**) Cu(I)-binding site of Cu_2Zn_2SOD.

Materials and methods

Cu–PuPy [*N*,*N*′-bis(2-pyridylmethylene)-1.4-butanediamine (*N*,*N*′,*N*″,*N*‴)–Cu(II)–di-perchlorate] was prepared according to the method of Felix *et al.* (1993). The diSchiff base ligand PuPy was prepared by the addition of 2 ml pyridine-2-aldehyde (21 mmol) to 1.05 ml putrescine (1,4-diaminobutane, 10.5 mmol) in 30 ml ethanol under constant stirring. During continued stirring overnight the solvent was allowed to evaporate and a yellow powder with the calculated molar ratios was obtained. Cu(I)–PuPy was formed by the addition of CuCl to the ligand under anaerobic conditions. Oxidation of this complex yielded a Cu(II) compound with the same EPR spectrum obtained from Cu(II)–PuPy which was prepared according to the method of Felix *et al.* (1993). Sodium α-oxyhyponitrite (Angeli's salt, $Na_2N_2O_3$), which served as a source for NO^-, was prepared by a modified method of Angeli (Hunt *et al.* 1962). Ethyl nitrate was replaced by *n*-propyl nitrate. Fresh solutions of $Na_2N_2O_3$ were prepared prior to each measurement in HEPES buffer (0.1 M, pH 12). All reactions were carried out in HEPES buffer (0.1 M, pH 7.0) to minimize the chelation of Cu(II).

Cu_2Zn_2SOD (bovine erythrocytes, 3175 units mg^{-1}) was from Serva (Heidelberg, Germany) and catalase (bovine liver, 65 000 units mg^{-1}) was from Boehringer Mannheim (Mannheim, Germany). Putrescine, pyridine-2-aldehyde and myoglobin were from Sigma (München, Germany), NO gas (purity > 99%) was from Merck (Darmstadt, Germany) and 3-morpholinosydnonimine hydrochloride (SIN-1) was a generous gift from Dr R. Greve, Cassella-Riedel Pharma GmbH (Frankfurt am Main, Germany). Aqueous (non-buffered) solutions at pH 5 had to be prepared freshly just before use.

Electronic absorption spectra and kinetic measurements were recorded on a Beckman Du 7400 photometer. EPR X-band spectra of Cu–PuPy, Cu_2Zn_2SOD and heme proteins were recorded on a Bruker ESP 300 E. The following parameters were chosen: modulation amplitude 5 G, modulation frequency 100 kHz, microwave power 20 mW and temperature 100 K. Aqueous solutions of Cu(II)–PuPy with 50% (v/v) ethylene glycol were used.

Results

The reduction of NO by Cu(I) compounds and the reverse reaction (reactions 3 and 4) were followed by monitoring the formation of MbNO (reaction 5). Upon the reaction with NO^-, the MetMb absorption band at 500 nm is red-shifted by 50 nm and a second band at 580 nm appears (Figure 2). The kinetics of the above mentioned reactions were measured at 580 nm.

During the measurements of the reaction between Cu(I)–PuPy and NO using MetMb as the detection system

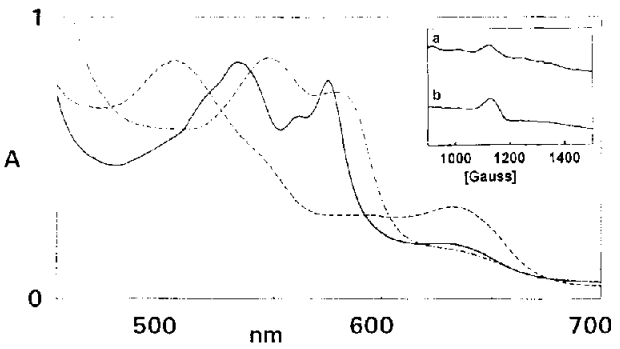

Figure 2. Electronic absorption of MetMb (– – –), MbNO (– · – · –) and MetMbNO (——). Protein concentration: 100 μM; HEPES buffer (0.1 M, pH 7). The inset shows the EPR spectra of MetMb (**a**) and MetMbNO (**b**). For details see Materials and methods.

for NO , a spectrum similar to that of MbNO was observed (Figure 2, spectrum of MetMbNO). It exhibits two extrema which were shifted by 5–20 nm to shorter wavelengths compared with that of MbNO and a shoulder at 560 nm was noticed. As this spectrum overlaps that of MbNO, the quantitative determination of the formation of NO^- with MetMb was unsuitable employing electronic absorption. After purging the solution of MetMbNO with nitrogen the electronic absorption spectrum of MetMb reappeared. The formation of MetMbNO (inset Figure 2b) was successfully deduced from its EPR spectrum which resembles that of MetMb (inset Figure 2a). These EPR measurements were performed at 100 K to distinguish between Fe(II) and Fe(III) myoglobin. For a detailed examination of the iron environment in the protein, EPR analysis has to be carried out at 4–20 K. An EPR spectrum identical to that of native Cu(II)–PuPy (Figure 3a) was measured after oxidation of Cu(I)–PuPy with NO or oxygen.

Substoichiometric addition of NO in an anaerobic aqueous solution to Cu(I)–PuPy resulted in the formation of the MbNO indicating that NO^- was produced. The nitrovasodilator SIN-1 was used a NO donor. During the decomposition both superoxide anions and NO are released (Feelisch 1991). Cu(II)–PuPy is reduced by superoxide to yield Cu(I)–PuPy. Reoxidation by oxygen or superoxide is possible. In the presence of NO, NO^- is formed which can be detected as MbNO. Heating the solution to 40 °C was appropriate to accelerate the decay of SIN-1 which normally would have taken several hours at 20 °C. Catalase (10 $\mu g\,ml^{-1}$) was added to scavenge disturbing hydrogen peroxide originating from the superoxide dismutation. The reactivity of Cu(II)–PuPy was compared with those of $CuSO_4$, Cu EDTA and Cu_2Zn_2SOD (Figure 4).

A clear increase of MbNO formation was observed in the presence of SOD which was even more pronounced in the case of Cu(II)–PuPy. However, no catalytic reactivity was seen with either copper compound with regard to the reduction of NO. Copper sulfate and Cu EDTA reduced NO derived from SIN-1 15 times slower compared with

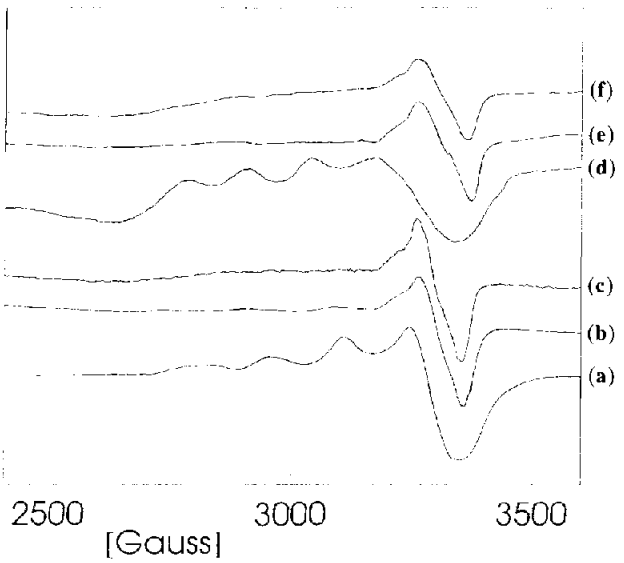

Figure 3. EPR spectra of Cu(II)–PuPy and Cu_2Zn_2SOD and derivatives after the reaction with SIN-1 and $Na_2N_2O_3$. (**a**) Cu(II)–PuPy (1 mM), (**b**) Cu(II)–PuPy (1 mM) + $Na_2N_2O_3$ (20 mM), (**c**) Cu(I)–PuPy (100 μM) + SIN-1 (10 mM), (**d**) intact Cu_2Zn_2SOD (0.1 $mg\,ml^{-1}$, 3.4 μM Cu), (**e**) Cu_2Zn_2SOD (0.25 $mg\,ml^{-1}$, 8.5 μM Cu) + $Na_2N_2O_3$ (200 μM), (**f**) Cu_2Zn_2SOD (1 $mg\,ml^{-1}$, 34 μM Cu) + SIN-1 (10 mM). For details see Materials and methods.

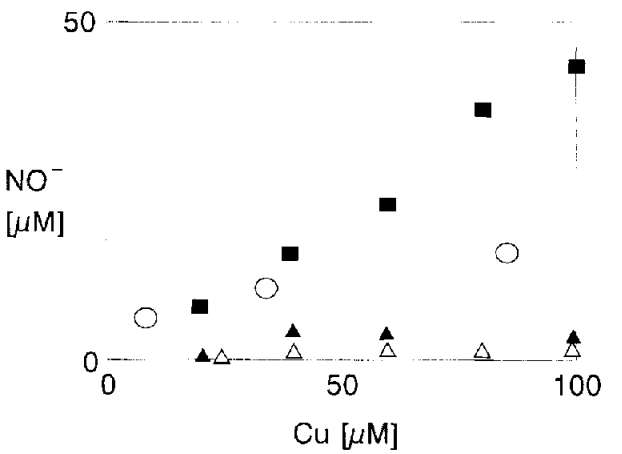

Figure 4. Reaction of Cu(II)–PuPy (■), $CuSO_4$ (▲), Cu EDTA (△) and Cu_2Zn_2SOD (○) with SIN-1 at different copper concentrations. SIN-1, 10 mM; MetMb, 0.1 mM; catalase, 10 $\mu g\,ml^{-1}$; HEPES buffer, 0.1 M; pH 7; T, 40 °C. Each point represents an average of three independent measurements. The reproducibility was better than 5%.

Cu(II)–PuPy and five times slower than Cu_2Zn_2SOD. The reaction solutions of Cu(II)–PuPy (Figure 3c) and Cu_2Zn_2SOD (Figure 3f) were examined with EPR spectroscopy. The hyperfine splittings characteristic for the copper environment in both the complex and the enzyme disappeared with increasing SIN-1 concentrations, and the main signals are shifted to higher field. These changes in the EPR properties indicate modifications of the copper environment which are probably due to reactions of

transient species formed from SIN-1 with the copper compounds. The ligands PuPy and EDTA showed no reaction with SIN-1 with respect to the formation of MbNO.

To measure the reaction of Cu(II)–PuPy with NO^-, Angeli's salt ($Na_2N_2O_3$) was used as a NO^- donor. Its aqueous solution is fairly stable under alkaline conditions (pH 12) while in neutral or acidic solutions it decomposes rapidly leading to HNO and NO^-.

$$H_2N_2O_3 \rightleftarrows HNO + HNO_2 \qquad (6)$$

NO^- is oxidized by Cu(II)–PuPy according to reaction (4). The samples were saturated with oxygen in a way that Cu(I)–PuPy could be reoxidized immediately leading to a catalytic reaction:

$$\text{Cu(I)–PuPy} \xrightarrow{O_2 \quad O_2^-} \text{Cu(II)–PuPy} \qquad (7)$$

The produced superoxide is efficiently eliminated by Cu–PuPy (Felix *et al.* 1993). The possible interferences of hydrogen peroxide during the dismutation of superoxide anions are excluded in the presence of catalase (10 $\mu g\,ml^{-1}$). In contrast of Cu_2Zn_2SOD, Cu(II)–PuPy was significantly more reactive to oxidase NO^- (Figure 5). The lowest effective concentration of Cu(II)–PuPy was 1 μM. It reacts up to three times faster with NO^- compared with aqueous Cu(II) ions and copper(II) EDTA. When copper was omitted the ligands, PuPy and EDTA did not react with $Na_2N_2O_3$.

Cu–PuPy and NO^- reacted catalytically in concentrations between 1 and 20 μM Cu. EPR measurements revealed changes in the shape of the spectrum of the copper complex depending on the ratio of copper to $Na_2N_2O_3$ which may originate from the reaction products of Angeli's salt. The signals were shifted to higher field and the hyperfine splittings disappeared with increasing $Na_2N_2O_3$ concentrations (Figure 3b). When Cu_2Zn_2SOD was exposed to Angeli's salt comparable results were obtained (Figures 3d and e).

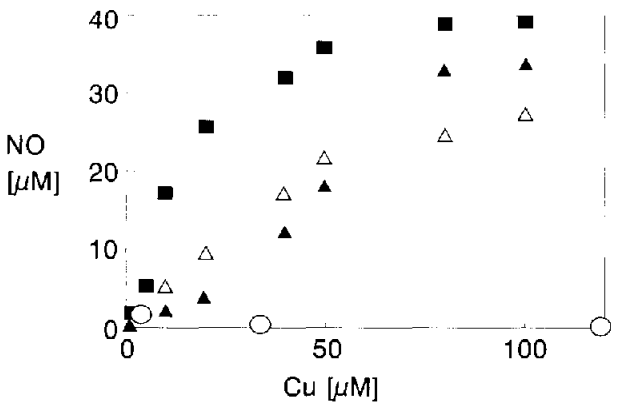

Figure 5. Reaction of Cu(II)–PuPy (■), $CuSO_4$ (▲), Cu EDTA (△) and Cu_2Zn_2SOD (○) with NO^- derived from $Na_2N_2O_3$ at different copper concentrations. $Na_2N_2O_3$, 1 mM; MetMb, 0.1 mM; catalase, 10 $\mu g\,ml^{-1}$; HEPES buffer, 0.1 M; pH 7; T, 20 °C. Each point represents an average of three independent measurements. The deviation is 7%.

Discussion

It could be demonstrated that Cu–PuPy reacts with chemically generated NO and NO^-. The heme protein myoglobin proved to be most convenient to detect the formation and/or reduction of NO and NO^-. In the presence of Cu(I)–PuPy, NO is reduced and converted into NO^-. Excessive NO caused the appearance of a new spectrum. The spectrum of MetMbNO overlaps with that of MbNO and obscures the quantitative measurement of the reaction kinetics. The NO of MetMbNO was separated by purging the solution with nitrogen and MetMb was obtained again. The EPR spectrum of MetMbNO is similar to that of MetMb. The NO-adduct of MetMb can lead to the reduction of MetMb to myoglobin and the oxidation of NO to NO^+ as reported for hemoglobin (Chien 1969). No MetMbNO was seen when the nitrovasodilator SIN-1 was used as a NO source.

A chemical modification of the copper environment in Cu–PuPy as well as in Cu_2Zn_2SOD following the reaction with SIN-1 was deduced from EPR measurements. This is probably due to by-products of the SIN-1 decay because these changes could be observed after the reaction with NO.

The binding of NO to iron and/or copper is proposed to be a further molecular basis of its biological action (Lancaster *et al.* 1990, Pellat *et al.* 1990, Stamler *et al.* 1992). With SIN-1 no catalytic reaction between the released NO and any of the copper compounds could be observed. Cu(I)–PuPy reduces NO more effectively than [CU(I)]–SOD. This phenomenon may be assigned to the protein moiety of the SOD which differs in the electrostatic charge at the protein surface and/or diminishes the funneling of NO to the active center. Unlike in Cu_2Zn_2SOD, Cu–PuPy is much less protected by the ligand allowing the convenient reaction of the substrate with the exposed copper site. Aqueous copper sulfate and Cu EDTA show no pronounced reactivity with NO. In these complexes, Cu(I) is unable to survive and dismutation to Cu(II) is the predominant step.

A catalytic reactivity between NO^- released from Angeli's salt ($Na_2N_2O_3$) and Cu(II)–PuPy could be observed in a concentration range between 1 and 20 μM Cu.

Cu_2Zn_2SOD shows a negligible reactivity towards NO^-. This may be explained by the molecular differences (Table 1) between NO^- and O_2^- and the above mentioned reasons concerning the reaction with NO derived from SIN-1. The observed changes in the EPR spectra of SOD and Cu–PuPy after the reaction with NO and its reduced form may be assigned to uncontrolled reactions of NO and NO^- with functional groups including amines and thiols. The direct reaction with copper has also to be taken into consideration (Stamler *et al.* 1992).

In conclusion, a stablization of NO through a reversible reduction to NO^- catalyzed by Cu(II)–PuPy or Cu_2Zn_2SOD seems to be of no considerable physiological

Table 1. Physical properties of nitric oxide, nitroxyl anion and superoxide anion

	NO	NO^-	O_2^-
Electron number	15	16	17
Charge	0	−1	−1
Bond length (Å)	1.15	1.26	1.33
Radical character	+	−	+

importance. The main effect of these copper compounds concerning the action of NO/EDRF is the accelerated catalysis of superoxide dismutation which prolongs the half-life of NO. Although a reversible reduction of NO to NO^- by Cu(I)–PuPy could be demonstrated, another nitrogen oxide seems to be the more likely intermediate form of NO. These results would support the suggestion of Furchgott (1988) and Schmidt *et al.* (1988) who favor nitrite to be a suitable and stable form of NO. It is formed upon neutralization from NO and it delivers NO when acidified. In addition to its well known superoxide dismutase (Felix *et al.* 1993) and Fenton reactivity (Miesel *et al.* 1990, Steinkühler *et al.* 1990, 1991), Cu–PuPy appears to be a remarkably efficient moderator of the biochemical reactivity of nitrogen monoxides.

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