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DETERMINATION OF THE SUPEROXIDE DISMUTASE-LIKE ACTIVITY OF CIMETIDINE-Cu(II) COMPLEXES

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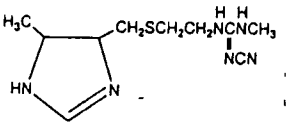
Using the direct method of pulse radiolysis to determine the superoxide dismutase like activity of copper(II) cimetidine complexes , it was found that the reaction rate constant with O_2^-, k_{cat}, was $(8.5 \pm 0.5) \times 10^8$ $M^{-1}s^{-1}$ independent of the cimetidine concentrations present in excess of 50–200 μM over the metal. The results suggest that either the 1:1 ligand to metal complex does not catalyze O_2^- dismutation at a comparable rate to that of the 2:1 complex, or that the stability constant of the last species is much higher than that determined earlier by Kimura *et al.*,[1] and only the 2:1 species is present in the solutions. With the indirect methods of cytochrome c and NBT for determining the ability of these complexes to catalyze O_2^- dismutation, these compounds exhibited a much lower SOD activity, and k_{cat} was determined to be $(5.0 \pm 0.3) \times 10^6$ and $(7.6 \pm 0.4) \times 10^7$ $M^{-1}s^{-1}$, respectively using the two assays.

KEY WORDS: SOD, cimetidine, copper, O_2^-, pulse radiolysis.

INTRODUCTION

Cimetidine (CM) is one of the most potent histamine H_2 receptor antagonists which is used for the treatment of peptic ulcer.[2] The drug is taken orally and reaches H_2 receptors via the blood stream, where micromolar levels of loosely bound cupric ions are present.[3]



Cimetidine

It was found that the drug readily reacts with copper(II) to produce the green cimetidine-Cu(II) complexes.[4] The complexation constants were determined to be $(3.02 \pm 0.05) \times 10^4$, $(2.35 \pm 0.05) \times 10^4$ and $(1.3 \pm 0.2) \times 10^9$ M^{-1} for (CM)Cu(II), $(CM)_2$Cu(II) and (CM)Cu(I), respectively.[1] The oxidation potential of (CM)Cu(I) was found to be 0.42 V,[1] similar to that of the superoxide dismutase (SOD).[5] Thus, using the cytochrome *c* assay for determining the ability of these complexes to catalyze O_2^- dismutation, it was found that these compounds have relatively high SOD activity as compared to other copper complexes, and that for (CM)Cu(I) $k_{cat} = 6.5 \times 10^6$ $M^{-1}s^{-1}$, an order of magnitude higher than that of $(CM)_2$Cu(II).[1]

One of the main reasons for the different SOD activity of copper compounds determined by pulse radiolysis as compared to that determined by indirect methods, e.g., cytochrome *c*, NBT, is due to a fast reoxidation of the cuprous complexes by molecular oxygen, which competes efficiently with the reoxidation by O_2^- in an indirect system.[6,7] (CM)Cu(I) is oxidized slowly in air to the green cupric complex,[1] and therefore we have chosen to determine k_{cat} of the cimetidine-copper(II) using the pulse radiolysis technique and compared the results to those obtained using the cytochrome *c* and NBT assays.

MATERIALS AND METHODS

All solutions were prepared using distilled water which passed through a millipore ultrapurification cation system. Sodium formate, sodium phosphate, cupric sulfate (Merck), cimetidine, nitro blue tetrazolium (NBT), cytochrome *c* Type VI from horse heart, xanthine, xanthine oxidase Grade I from butter milk (Sigma) were all used as purchased.

Pulse radiolysis experiments were carried out with a Varian 7715 accelerator as described previously.[8] Superoxide radicals were generated in O_2-saturated aqueous solutions containing 50 mM formate at pH 7 (1 mM phosphate buffer) according to a well established mechanism,[9] and the concentration of O_2^- generated initially was about 15 μM as determined from its absorbance ($\varepsilon_{245} = 2350\,M^{-1}cm^{-1}$).[9]

The cytochrome *c* and NBT assays were performed in 3 ml of 2 mM sodium phosphate buffer at pH 7 in the absence of EDTA. The reaction mixture contained 10 μM cytochrome *c* or 100 μM NBT, 50 μM xanthine and sufficient xanthine oxidase to produce about 1.2 μM O_2^-/min. The formation of ferrocytochrome *c* and monoformazan were followed at 550 and 560 nm, respetivley. Under these conditions the rate constants of the reaction of ferricytochrome *c* and NBT with O_2^- were determined to be $1.1 \times 10^6\ M^{-1}s^{-1}$,[10] and $5.88 \times 10^4\ M^{-1}s^{-1}$,[11] respectively. Our experimental conditions are somewhat different than those originally used with these assays.[12,13] Typically, in the cytochrome *c* system the pH is adjusted to 7.8 (50 mM phosphate buffer)[12] and in the NBT system to 10.2 (50 mM carbonate buffer),[13] and in both assays the reaction mixture also contained 0.1 mM EDTA.[12,13] Under these conditions the rate constant of the reaction of ferricytochrome *c* with O_2^- decreases to $2.6 \times 10^5\ M^{-1}s^{-1}$ due to ionic strength effect.[10]

RESULTS

The decay of the absorbance of O_2^- was followed at 240–270 nm, and the addition of up to 200 μM of cimetidine alone did not alter the observed decay rate, which was first order and not a second order, due to catalytic impurities present in the solutions. We were unable to use EDTA, as it would sequester the copper out of the cimetidine complexes. Thus, cimetidine does not react with O_2^- nor does it form complexes with metal impurities having catalytic properties.

Under the condition where $[Cu(II)]_0 < [O_2^-]_0$ in the presence of either 50 or 200 μM cimetidine, the decay of the abosrbance of O_2^- was accelerated followed pseudo-first-order kinetics. The observed pseudo-first-order rate constant (k_{obs}) was linearly dependent on $[Cu(II)]_0$, and the second order rate constant (k_{cat}) was obtained by plotting k_{obs} versus $[Cu(II)]_0$, $k_{cat} = (8.5 \pm 0.5) \times 10^8\ M^{-1}s^{-1}$ (Figure 1). It was

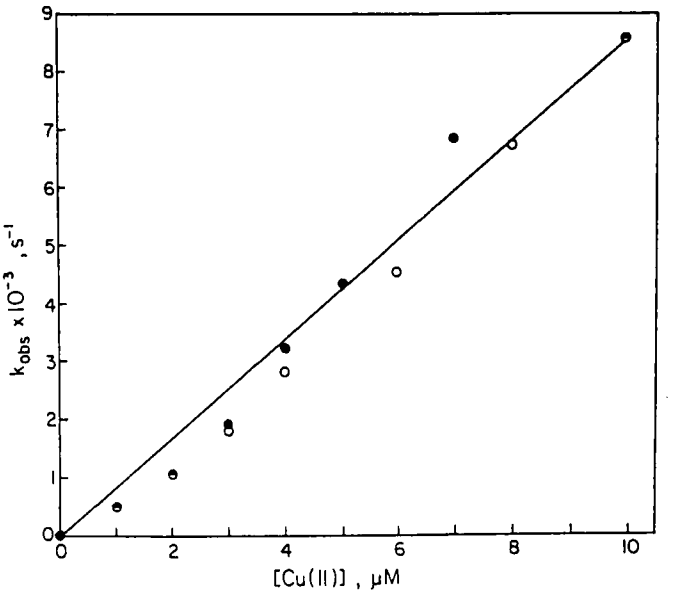


FIGURE 1 The pseudo-first-order rate constant as a function of $[Cu(II)]_0$ at constant concentrations of ● — 50 μM and ○ — 200 μM cimetidine. The solutions were O_2-saturated and contained 50 mM formate at pH 7.

found that within experimental error, k_{cat} was independent of the cimetidine concentrations (Figure 1).

With cytochrome *c* and NBT assays, the initial rate of the formation of the absorbance of ferrocytochrome *c* or monoformazan in the absence of Cu(II) (V_0) and

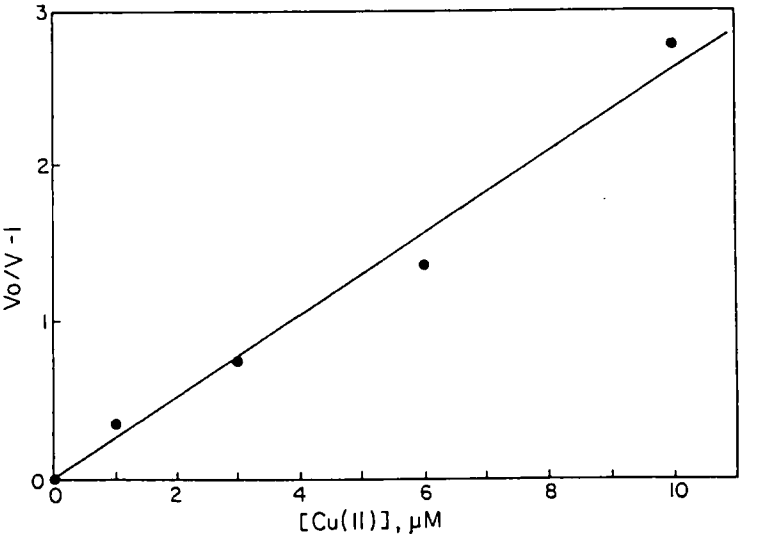


FIGURE 2 $V_0/V_c - 1$ as a function of $[Cu(II)]_0$. The solutions contained 200 μM cimetidine, 10 μM cytochrome *c*, 50 μM xanthine at pH 7 and sufficient xanthine oxidase that generated about 1.2 μM O_2^-/min.

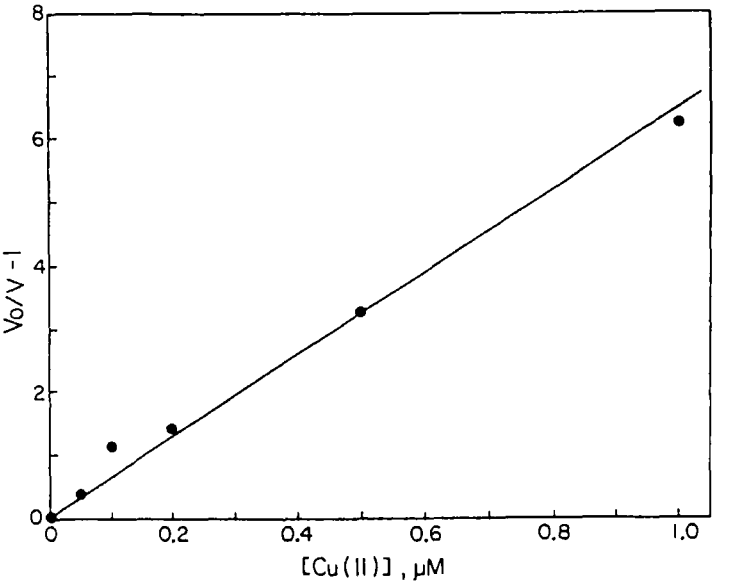


FIGURE 3 $V_0/V_c - 1$ as a function of $[Cu(II)]_0$. The solutions contained 200 μM cimetidine, 100 μM NBT, 50 μM xanthine at pH 7, and sufficient xanthine oxidase that generated about 1.2 μM O_2^-/min.

in its presence (V) were measured in solutions containing 200 μM cimetidine. Cimetidine alone did not influence the initial rate. Thus, by plotting ($V_0/V - 1$) versus $[Cu(II)]_0$, k_{cat} can be calculated,[6] and in our experiments k_{cat} was found to be $(5.0 \pm 0.3) \times 10^6$ and $(7.6 \pm 0.4) \times 10^7\ M^{-1}s^{-1}$, respectively from the cytochrome c and NBT methods (Figures 2 and 3).

DISCUSSION

We have found with the use of pulse radiolysis technique that the copper cimetidine complexes catalyze O_2^- dismutation very efficiently at pH 7 with $k_{cat} = (8.5 \pm 0.5) \times 10^8\ M^{-1}s^{-1}$, independent of the cimetidine concentration when present in excess of 50–200 μM over the metal. In Table I the distribution of the different species present in the solutions is calculated using Kimura *et al.*,[1] values for the complexation constants. From the values given in Table I, taking into account that free cupric ions catalyze O_2^- dismutation with $k_{cat} = (1.9 \pm 0.2) \times 10^9\ M^{-1}s^{-1}$

TABLE I
The distribution of the species present in the solutions containing excess concentrations of 50 and 200 μM of the ligand over the metal assuming $K(CuL) = 3 \times 10^4\ M^{-1}$ and $K(CuL_2) = 2.35 \times 10^4\ M^{-1}$.

L μM	Cu	CuL	CuL_2	$k_{cat} \times 10^{-9}$ $M^{-1}s^{-1}$
0	100%	—	—	1.90
50	24%	35%	41%	0.85
200	3%	17%	80%	0.85

under the experimental conditions,[6] we conclude that either the stability constant of $(CM)_2Cu(II)$ is much higher than $2.35 \times 10^4\ M^{-1}$,[1] and practically only $(CM)_2Cu(II)$ is present in the solutions and its $k_{cat} = 8.5 \times 10^8\ M^{-1}S^{-1}$, or that if the values determined by Kimura *et al.*,[1] are correct then k_{cat} of (CM)Cu(II) is much lower than that of $(CM)_2Cu(II)$, and it can be neglected. In this case, taking into account the presence of free cupric ions in the solutions, we obtain $k_{cat} = (9.8 \pm 0.5) \times 10^8\ M^{-1}s^{-1}$ for $(CM)_2Cu(II)$.

According to Kimura *et al.*[1] the active species is most likely the 1:1 complex and the Cu(I) $\rightleftharpoons$ Cu(II) conversion is reversible without decomposition of the complexes. If this is the case, assuming that during the catalysis copper oscillates between Cu(II) and Cu(I), k_{cat} of $(CM)_2Cu(II)$ and of $(CM)_2Cu(I)$ should be the same, independent whether one starts with $(CM)_2Cu(II)$ or (CM)Cu(I). Beside the fact that Kimura *et al.* determined different values of k_{cat} for (CM)Cu(I) and for $(CM)_2Cu(II)$, they also used 0.1 mM EDTA in the reaction mixture. Thus, their results seem to be quite surprising as EDTA is a very strong copper chelator, and EDTA-Cu(II) does not catalyze O_2^- dismutation.[14]

With the cytochrome *c* assay we determined $k_{cat} = (5.0 \pm 0.3) \times 10^6\ M^{-1}s^{-1}$ for cimetidine-Cu(II) in the presence of excess of 200 μM cimetidine and in the absence of EDTA. This value is in agreement with that obtained by Kimura *et al.*[1] for (CM)Cu(I) in the presence of 0.1 mM EDTA. When we added only 10 μM EDTA to the reaction mixture, we could not find any SOD activity as expected and in conflict to earlier studies.[1]

Although it was reported that (CM)Cu(I) is oxidized slowly by oxygen, which is a prerequisit to get similar values of k_{cat} using indirect and direct methods, the SOD activity of the cimetidine-Cu(II) as determined by pulse radiolysis was much higher than that determined by the NBT and the cytochrome *c* assays. In fact, the difference in k_{cat} obtained by pulse radiolysis, NBT and cytochrome *c* assays resembles that of other copper complexes which in their reduced form are reoxidized much faster by oxygen.[6,7] Therefore, we have to assume that either the redox potential of $(CM)_2Cu(II)$ is much lower than that determined for (CM)Cu(I), and its reduced form reacts rapidly with oxygen, or that some other side reactions interfere with the determination using indirect methods.[6,15] The copper complex may interfere with the generating system, but this possibility was ruled out as the same results were obtained when the xanthine/xanthine oxidase system was replaced by gamma-radiolysis.[6] As copper alone has similar SOD activity to that of cimetidine-Cu(II) using the cytochrome *c* and NBT assays,[6,14,16] the possibility that the metal is sequestered by the dye exists. The cuprous ions and cuprous cimetidine may react similarly with the dye,[6,15] in this case the measured SOD activity would probably be the same. We were unable to follow this rection as the cuprous cimetidine complex is insoluble species in water. Its maximum solubility in 50 mM phosphate buffer at pH 7.8 is only 34 μM as estimated by atomic absorption spectroscopy method.[1] It is also possible that NBT and cytochrome *c* form ternary complexes with cimetidine-Cu, which have different kinetic properties than those of cimetidine-Cu. All the factors that may affect the determination of the SOD activity of a coppr compound using indirect methods were discussed elsewhere in more details.[6,15,17]

Acknowledgements

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