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Reactivity of Active Centre Analogues of Cu_2Zn_2-superoxide Dismutase During the Aqueous Decay of K_3CrO_8*

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Abstract

The aqueous decay of K_3CrO_8 was used to compare the reactivity of Cu_2Zn_2-superoxide dismutase and two active centre analogues where the first shell atoms around the copper are four unsaturated nitrogens. Unlike the acetate- or biuret-type Cu(II) chelates these di-Schiff base complexes had an identical reactivity compared to that of the intact enzyme. Nanomolar concentrations of copper coordinated in these complexes were sufficient to inhibit the K_3CrO_8 induced chemiluminescence by 50%. They were also able to inhibit the hyaluronic acid degradation successfully by 27%.

Introduction

In contrast to the many known low M_r Cu chelates where Cu(II) is found in an acetate or biuret structure [1] a genuine active site analogue of Cu_2Zn_2-superoxide dismutase has been reported [2, 3]. As in the native enzyme the first shell atoms around the copper are four unsaturated nitrogens. Unlike the imidazolate nitrogens of the protein backbone these coordinating nitrogens are derived from the di-Schiff bases of pyridine-2-aldehyde, imidazole-2-aldehyde and 1,4-diamino-butane, respectively. The butyl moiety ascertains the flexibility in a way similar to the situation found in the intact enzyme.

Nevertheless, all these low M_r Cu complexes are known to be active in an oxygen induced inflammatory process [4]. They may inhibit the initiation or react with the many different excited oxygen species or other transient radical compounds [5]. The inhibitory action of acetate or biuret type Cu chelates do not allow a decision whether or not the complete Cu complex or dissociated Cu(II) may be attributed to the observed phenomenon. These complexes do not survive biological Cu chelators including serum albumin.

Much to our surprise the structural active centre analogues are fairly stable in the presence of serum albumin. Although the redox properties of the di-Schiff base complex are virtually identical to those of Cu_2Zn_2-superoxide dismutase [3] a limited enzymic activity was noticed using the nitro tetrazolium blue reductase assay where $\cdot O_2^-$ is generated by the xanthine/xanthine oxidase system. It was of interest to compare the superoxide dismutase activity of some of these Cu complexes and the native copper enzyme employing an assay omitting any of the beforementioned compounds. The earlier described CrO_8^{3-} decay [6] proved most appropriate to shed some light on this phenomenon.

Experimental

Chemicals

All chemicals were of analytical purity or better. 3-Aminophthalhydrazide (luminol) was from Aldrich, Milwaukee. Catalase from bovine liver was obtained from Boehringer, Mannheim. Bovine serum albumin, diethylenetriaminepentaacetate (DTPA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (HEPES) and nitrotetrazolium blue chloride (NBT) were from Serva, Heidelberg; hyaluronic acid, putrescine and mannitol from Sigma, St. Louis. Deionized water was quartz distilled and additionally purified on a Millipore Milli Q water purification system. The conductivity was 50 nS. Cu_2Zn_2-superoxide dismutase was isolated from bovine erythrocytes after heating the haemolysate to 75 °C [7]. K_3CrO_8 was prepared as earlier described [8]. {[*N*,*N'*-Bis(2-pyridylmethylene-1,4-butanediamine]-(*N*,*N'*,*N''*,*N'''*)}Cu(II)diperchlorate [Cu-Pu(Py)$_2$] and {[1.8-di(2-imidazoyl)-2.7-diazaoctadiene-1.7]-(*N*,*N'*,*N''*,*N'''*)}Cu(II)diperchlorate [Cu-Pu(Im)$_2$] were synthesized as described in refs. 3 and 4 and Cu(II)-desferrithiocin according to ref. 9. Desferrithiocin was kindly donated by Ciba Geigy AG, Basel.

CrO_8^{3-} Decay

During the decay of CrO_8^{3-}, singlet oxygen and many other excited oxygen species are transiently

*Abbreviations used: Cu-Pu(Py)$_2$: {[*N*,*N'*-bis(2-pyridylmethylene)-1,4-butanediamine]-(*N*,*N'*,*N''*,*N'''*)}Cu(II)diperchlorate, Cu-Pu(Im)$_2$: {[1.8-Di(2-imidazoyl)-2.7-diazaoctadiene-1.7]-(*N*,*N'*,*N''*,*N'''*)}Cu(II)diperchlorate.

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formed [6]. They can be traced by measuring the chemiluminescence caused via secondary reactions including a 2+4-cycloaddition of these oxygen species to the heteroaromatic system of the added luminol. N_2 is cleaved and photons are emitted [10]. They were detected in a Lumac luminometer Biocounter 2010. There is a competitive reaction of the transiently formed oxygen species between both the chelated Cu(II) and luminol. As a consequence the yield of photons is diminished. For comparative reasons the concentration of chelated copper was quantified which was required to inhibit the initial chemiluminescence by 50%.

Hyaluronic Acid Depolymerization

Hyaluronic acid depolymerization was measured in the presence of K_3CrO_8 using a Schott Micro-KPG-Ubbelohde viscosimeter. The penetration diameter of the glass capillary was 0.6 mm. The viscosity was controlled for 20 min. The decrease in relative viscosity was obtained graphically. The experimental conditions were: 0.9 mg/ml (bovine) hyaluronic acid and 50 μM of different Cu chelates were combined at 25 °C. The reaction was started with 150 μM K_3CrO_8 in oxygen saturated 20 mM HEPES-buffer at pH 7.8. The total volume was 2 ml.

Results and Discussion

CrO_8^{3-} Decay and Chelated Copper

The reactivity of Cu_2Zn_2-superoxide dismutase, two active centre analogues of this copper protein and some Cu(II) chelates involving C=O or C–O^- coordination were compared using the aqueous decay of K_3CrO_8 in the absence of competive Cu(II) chelates. The CrO_8^{3-} decay into transiently formed excited oxygen species was monitored by chemiluminescene in the presence of luminol (Fig. 1, Table 1).

Additions of 10 mM mannitol for the possible quenching of ·OH as well as 10 mM formate to increase the yield of $\cdot O_2^-$ did not affect the chemiluminescence. Thus, secondary reactions from $\cdot O_2^-$ and ·OH leading to the observed chemiluminescence must be discarded. Singlet oxygen and/or transient radical species of chromium peroxide have to be attributed to the observed emission of photons. All ligands of the employed Cu(II) chelates including putrescine, pyridine-2-aldehyde, imidazole-2-aldehyde, salicylate, serine and desferrithiocin did not affect the chemiluminescence until 10 μM were reached. It was intriguing to notice the identical reactivity of Cu-Pu$(Py)_2$ compared to the intact Cu_2Zn_2-superoxide dismutase. Only 3 nM of Cu-Pu$(Py)_2$ were required for 50% inhibition of the initial chemiluminescence. When pyridine was replaced by imidazole 10 times higher concentrations of the Cu(II) complex became necessary. Compared to Cu$(sal)_2$, Cu$(ser)_2$, $CuSO_4$ and Cu-desferrithiocin the reactivity of either active centre analogue was remarkably pronounced. Sequential additions of 15 μM K_3CrO_8 three times each to the same incubation mixture containing Cu-Pu$(Py)_2$, Cu-Pu$(Im)_2$ or Cu_2Zn_2-superoxide dismutase did not change the rate of inhibition. In the presence of Cu(II)salicylate an irregular type of kinetics can be observed (Fig. 2).

TABLE 1. Comparison of the K_3CrO_8-dependent luminol-chemiluminescence between various low M_r copper complexes and Cu_2Zn_2-superoxide dismutase[a]

Cu chelate	nM Cu(II) for 50% inhibition of photonemission	Superoxide dismutase units
Cu_2Zn_2-superoxide dismutase	3	1
Cu-Pu$(Py)_2$	3	1
Cu-Pu$(Im)_2$	32	10
Cu-(salicylate)$_2$	300	97
Cu-(serine)$_2$	950	306
$CuSO_4$	1000	323
Cu-desferrithiocin	3400	1097

[a]For experimental details see legend to Fig. 1.

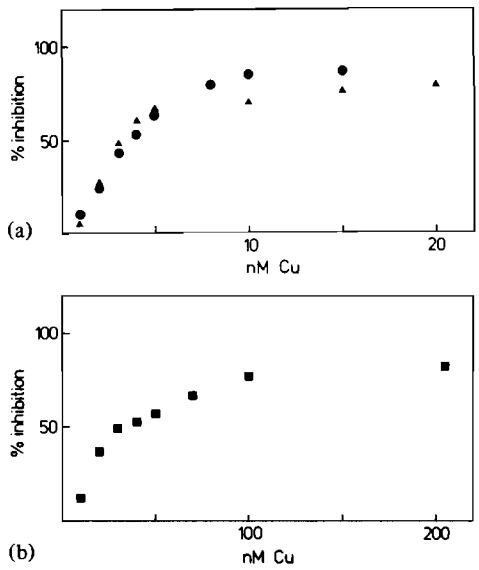

Fig. 1. Inhibition of luminol-chemiluminescence in the presence of K_3CrO_8 and active center analogues of Cu_2Zn_2-superoxide dismutase: a, ▲ Cu-Pu$(Py)_2$, ● Cu_2Zn_2SOD; b, ■ Cu-Pu$(Im)_2$. 1.5 ml contained: 200 μM luminol in O_2-saturated HEPES buffer 100 mM pH 7.8. Different concentrations of chelated copper. The reaction was started by the addition of 15 μM K_3CrO_8 in 10 mM NaOH. The overall time for the completed luminol-mediated chemiluminescence was measured in a Lumac 2010 luminometer at 25 °C. Each test was repeated three times. The reproducibility was better than ±5%.

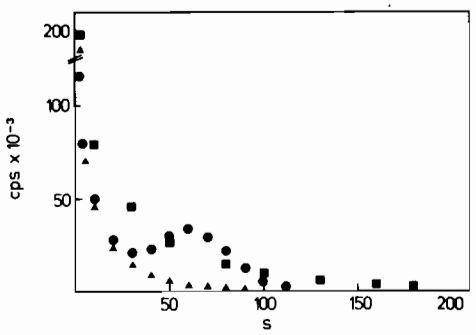

Fig. 2. Kinetics of the repeated luminol-mediated chemiluminescence in the presence of 300 nM Cu(salicylate)$_2$. First start with 15 μM K_3CrO_8 (▲), two subsequent additions of 15 μM K_3CrO_8 (●), Cu(salicylate)$_2$ omitted (■). For further experimental details see legend to Fig. 1.

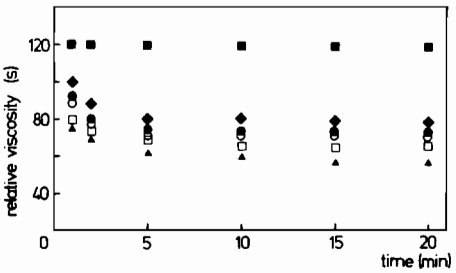

Fig. 3. Time-dependent inhibition of the depolymerization of hyaluronic acid by chelated copper and catalase. ▲Control without and with diethylenetriaminepentaacetate to scavenge spurius transition metals, ■ K_3CrO_8 omitted, ● Cu-Pu(Py)$_2$, ○ Cu-Pu(Im)$_2$, □ $CuSO_4$ respectively Cu(Sal)$_2$, ◆ 1 μg/ml catalase. 2 ml contained: 0.9 mg/ml hyaluronic acid in O_2-saturated HEPES buffer 20 mM pH 7.8. Chelated copper in 50 μM concentrations. The reaction was started by the addition of 150 μM K_3CrO_8. The decline in viscosity was measured in a Schott Mikro-KPG-Ubbelohde viscosimeter (ϕ 0.6 mm) over a period of 20 min at 25 °C. The reproducibility was ±7%.

A destruction of the salicylate ligand was concluded. Eventually there remains the type of decay similar to that observed in the presence of $CuSO_4$.

CrO_8^{3-}-induced Depolymerization of Hyaluronic Acid

The presence of CrO_8^{3-} caused a marked depolymerization of hyaluronic acid (Fig. 3). The most likely oxygen species responsible for the observed reactivity was attributed to be ·OH or transient type peroxochromate. Catalase known to react with ·OH at a calculated rate of $k_2 = 2.6 \times 10^{11}$ M^{-1} s^{-1} [11, 12] diminished the breakdown by 38%. In the presence of Cu_2Zn_2-superoxide dismutase an insignificant suppression by 6% was seen. This value was within the standard deviation. $CuSO_4$ and Cu salicylate were able to inhibit significantly although only 13% were detected. A marked suppression of hyaluronic acid degradation by 22% and 27% was noticed in the presence of the active centre analogues Cu-Pu(Im)$_2$ and Cu-Pu(Py)$_2$, respectively.

Conclusions

There are many different superoxide dismutase assays in which uncontrolled metal chelation of the different constituents including enzymes, flavins, serum albumin or metal scavengers like EDTA is observed. Thus, the actual reactivity of superoxide dismutase mimicks is quite frequently obscured. Similar to pulse radiolytic measurements [13, 14] the K_3CrO_8 decay is a suitable tool to compare the efficacy of the intact Cu enzyme with that of the active centre analogues. Earlier reports from this laboratory [6] have shown that acetate- or biuret-type Cu(II) chelates reacted insignificantly during CrO_8^{3-} decay. By way of contrast, the two di-Schiff base active centre analogues displayed a surprisingly identical activity in the chemiluminescence measurements. As they are also marked inhibitors of the hyaluronic acid depolymerization their possible use as potent antiinflammatory compounds will be awaited with great interest.

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