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CHEMILUMINESCENCE ASSAYS OF Cu_2Zn_2 SUPEROXIDE DISMUTASE MIMICKING Cu-COMPLEXES

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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%.

Furthermore, a lucigenin amplified chemiluminescence assay based on isolated polymorph nuclear leucocytes in the absence and presence of whole, unseparated blood was developed and successfully employed. $CuPu(Im)_2$ and $CuPu(Py)_2$ equivalent to 0.5 and 0.8 SOD units, only, were required to inhibit the photon emission by 50% in the absence of bovine serum albumin. Even in the presence of 600 μM albumin mimicking the competitive copper chelation in biological fluids $Cu\text{-}Pu(Py)_2$ and $CuPu(Im)_2$ remained active, whereas the carboxylate- and biuret type chelates $Cu(Sal)_2$ and $Cu(Ser)_2$ reacted like $CuSO_4$ The same reactivity of these low M_r SOD mimics was seen in human blood.

KEY WORDS: SOD mimicks, chemiluninescence, low M_r Cu-chelates, luminol, lucigenin, K_3CrO_8, TPA-activated PMNs.

ABBREVIATIONS: SOD Cu_2Zn_2 superoxide dismutase (EC. 1.15.1.1), TPA 12-o-Tetra-decanoylphorbol-13-acetate, PMN Polymorph nuclear leucocyte, $CuPu(Py)_2$ {[N,N'-bis (2-pyridylmethylene)-1,4-butanediamine (N,N', N'', N''')}-copper(II), $CuPu(Im)_2$ {[1,8-Di(2-imidazolyl)-2,7-diazaoctadiene-1.7]-(N,N', N'', N''')}-copper(II), Cu-Thiocin 1:1 copper complex with desferrithiocin isolated from Streptomyces antibioticus, HEPES N-(2-Hydroxyethyl) piperazine-N'-[ethanesulfonic acid]), NBT Nitro Blue Tetrazolium chloride, [I_{50}] concentration of chelated copper required for a 50% inhibited photon emission.

INTRODUCTION

By way of 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 designed and successfully employed.[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.

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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[4] proved most appropriate to shed some light on this phenomenon.

Superoxide is known to be a weak oxidizing agent.[5,6] It rather represents the intermediate in many a redox reaction yielding $\cdot OH$, H_2O_2 and possibly singlet oxygen[7,8] which belong to the more potent oxygenating agents. These reactive species originating from stimulated phagocytes react with substrates to generate short lived products notably dioxetanes known to disintegrate into electronically excited carbonyles which emit photons upon relaxation.[9] This native low level chemiluminescence, the energy endproduct of activated phagocytes, can be amplified more than 10^3 times employing luminol or lucigenin as co-substrates.[8] Luminol largely detects H_2O_2, whereas lucigenin reacts with $\cdot O_2^-$ to form two N-methylacridone molecules, one of which is electronically excited.[10,11]

In the present assay system the lucigenin amplified chemiluminescence is competively inhibited by Cu_2Zn_2 superoxide dismutase and/or its low M_r mimics. The concentration required to inhibit the phagocytic production of $\cdot O_2^-$ by 50% [I_{50}] was monitored by observing the photon emission in a luminometer. The mode of emission was recorded and the data compared equivalent to Cu_2Zn_2 superoxide dismutase units.

METHODS

CrO_8^{3-}-decay

During the decay of CrO_8^{3-}, singlet oxygen and many other excited oxygen species are transiently formed.[4] 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 and photons are emitted.[12] 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%.

Analytical

The deionized water used was quartz-distilled and additionally purified with a Millipore water purification system. The conductivity was less than 0.05 μS.

Copper concentrations were determined on a Perkin Elmer atomic absorption unit equipped with a graphite furnace.

Electron paramagnetic resonance (EPR) spectra were run on a Varian E 109 spectrometer at modulation amplitude 12.5 G, modulation frequency 100 kHz, microwave power 20 mW, microwave frequency 9.24 GHz, temperature 77 K.

The chemiluminescence measurements were carried out in a six-channel Biolumat LB 9505 C luminometer (Berthold, Wildbad, FRG) furnished with an Epson computing unit. Each determination was performed in duplicate. The reproducibility was better than $\pm 4\%$. Chemiluminescence in the presence of the different Cu-chelates was expressed as a percentage of the chemiluminescence generated by the same cell suspension omitting the Cu-complexes. Dose-response curves were recorded over 10 minutes each and the total photon emission was integrated.

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 chemiluminescence in the presence of luminol (Table I).

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.

Reactivity of SOD mimics on activated polymorph leucocytes

When 1×10^6 porcine PMNs/ml were incubated with 100 nM CuPu(Py)$_2$, Cu-

TABLE I

Comparison of the K_3CrO_8-dependent luminol-chemiluminescence between various low M_r copper complexes and Cu_2Zn_2 superoxide dismutase

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

TABLE II

Concentrations of differently chelated copper required to inhibit the oxidative burst dependent superoxide production of 1×10^6 porcine PMNs by 50%.

Copper chelate	nM Cu(II) for 50% inhibited photon emission	units related to SOD
$CuSO_4$	125	1.25
$Cu(Serinate)_2$	90	0.9
$Cu(Salicylate)_2$	80	0.8
Cu-Thiocin	200	2
$CuPu(Im)_2$	45	0.45
$CuPu(Py)_2$	75	0.75
Cu_2Zn_2 suproxide dismutase	100	1

$Pu(Im)_2$, $Cu(Sal)_2$, $Cu(Ser)_2$, Cu-Thiocin or Cu_2Zn_2 superoxide dismutase in the absence of serum albumin a pronounced inhibition of the lucigenin mediated photon emission of TPA activated porcine PMNs was seen (Table II). The most distinct inhibition was noticed in the presence of $CuPu(Im)_2$ (80%) and $CuPu(py)_2$ (70%). The reaction of the biuret type complex $Cu(Ser)_2$ was indistinguishable from that of $Cu–SO_4$.

In the absence of albumin $CuPu(Im)_2$ and $CuPu(Py)_2$ in concentrations equivalent to 0.5 or 0.8 SOD units were needed. Likewise the carboxylate and biuret type complexes $Cu(Sal)_2$ and $Cu(Ser)_2$ were 10–20% more active than SOD, whereas in the presence of $CuSO_4$ and Cu-Thiocin 3–4 times higher concentrations were necesary to observe the same inhibition obtained with $CuPu(Im)_2$ and $CuPu(Py)_2$. Addition of 20 μM of the corresponding ligands (putrescine, pyridine-2-aldehyde, imidazole-2-aldehyde, salicylate, serine, desferrithiocin) showed no detectable inhibition.

To evaluate the activity of the copper chelates in the presence of competitive biological chelators, the incubation medium was titrated with bovine serum albumin until a concentration of 600 μM similar to physiological conditions was reached. Dose-response experiments for all complexes were performed using 4 different albumin concentrations. The [I_{50}] was evaluated graphically (Table II).

Increasing albumin concentrations did not affect the reactivity of intact Cu_2Zn_2 superoxide dismutase. This can be explained by the electrostatic facilitation by a

TABLE III

Comparison of differently bound copper in its inhibitory reactivity on 2×10^4 human PMNs/ml in whole, unseparated blood. 1 ml contained: 100 μM lucigenin, human blood 1:250 in O_2-saturated HEPES buffered saline 50 mM pH 7.4 supplemented with 1 mM $MgSO_4$ and $CaCl_2$ each plus 5.6 mM glucose. After 10 minutes of preincubation at 37°C the phagocytes were activated with 250 nM TPA.

Copper chelate	nM Cu(II) for 50% inhibited photon emission	units relative to SOD
$Cu(Serinate)_2$	2000	5
$CuSO_4$	2000	5
Cu-Thiocin	2000	5
$Cu(Salicylate)_2$	1600	4
$CuPu(Im)_2$	400	1
$CuPu(Py)_2$	350	0.9
Cu_2Zn_2 superoxide dismutase	400	1

focused electrostatic field mainly caused by basic amino acids that cooperatively direct $\cdot O_2^-$ into the catalytic center.[13] This stability of the superoxide dismuting activity in the presence of increasing albumin concentrations could not be shown with any of the low M_r-mimics tested. 10 μM $CuPu(Py)_2$ or 55 μM $CuPu(Im)_2$ were needed for a half-maximal inhibition of the phagocytic response at a physiological concentration of albumin whereas $Cu(Sal)_2$ (I_{50} = 70 μM) and $Cu(Ser)_2$ (I_{50} = 90 μM) fairly behaved like $CuSO_4$ (I_{50} = 110 μM) (Figure 5). Cu-Thiocin, where Cu is complexed with 1 thiazolin-nitrogen, 1 oxygen of phenolic origin and 1 carboxylate oxygen[14] required 70 μM for the same amount of inhibition. In the presence and absence of serum albumin all ligands up to 200 μM were ineffective.

In order to exclude possible interferences with other blood cells during the phagocytic burst the activity of the complexes was examined in whole, unseparated human blood which contained 2×10^4 PMNs/ml. Much to our surprise the concentration of Cu_2Zn_2 superoxide dismutase had to be 3.2-fold to measure the same inhibition as seen with isolated porcine PMNs under the same conditions (Table III).

Unlike $Cu(Sal)_2$, Cu-Thiocin and $Cu(Ser)_2$ where 4–5 times higher concentration became necessary $CuPu(Py)_2$, $CuPu(Im)_2$ and SOD showed fairly identical I_{50}-values (350–400 nM). These data are of striking similarity to those found with isolated PMNs supplemented with 3 μM albumin.

Acetate and biuret-type complexes do not survive competitive biological chelators.[15] In this context it was of interest as to whether or not the di-Schiff-base type Cu-chelates though albumin resistant would be able to resist the presence of activated PMNs. Consistent with the cyclic voltammetry data[3] and the present chemiluminescence measurements $CuPu(Py)_2$ tolerated these activated cells while $CuPu(Im)_2$ was readily oxidized. This was deduced by the biuret type Cu-EPR signal which was obtained after 10 minutes using the latter complex. It should be emphasized that Cu_2Zn_2 superoxide dismutase remained intact when exposed to these activated PMNs.

CONCLUSIONS

Similar to pulse radiolytic measurements[16,17] 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[4] 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.

Using activated polymorph leucocytes only $CuPu(Im)_2$, and Cu-Thiocine survived albumin and remained active with regard to their superoxide dismutase activity against these activated cells. The amplified chemiluminescence assay of isolated PMNs, macrophages or in whole blood has become a clinical and laboratory routine procedure to identify and characterize test compounds for their antiinflammatory reactivity.[18] The most effective nonsteroidal antiinflammatory drugs required concentrations of 10–100 μM for a 50% inhibition in the lucigenin-amplified PMN assay without albumin.[15] 25–250 times lower concentrations are sufficient when the di-Schiff-base complexes low M_r mimics $CuPu(Py)_2$ and $CuPu(Im)_2$ were present.

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