SINGLET OXYGEN AND SUPEROXIDE DISMUTASE (CUPREIN)

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SUMMARY: Singlet oxygen of elongated lifetime was generated photochemically in ^2H_2O-solutions in the presence of molecular oxygen and methylene blue. The oxidation of 1,3-diphenylisobenzofuran by singlet oxygen was both significantly and specifically inhibited by superoxide dismutase. No such inhibition was observed using the apoprotein, $CuSO_4$, $Cu(tyr)_2$, $Cu(his)_2$, $Cu(lys)_2$, Cu-serum albumin, hemocyanin, ceruloplasmin, diamine oxidase, horseradish peroxidase, catalase and hemoglobin. Cyanide and azide added in approximately equimolar concentrations proved to be potent inhibitors of this biochemical action of cuprein. The extraordinary stability of superoxide dismutase before and after singlet oxygen treatment was demonstrated by the identical parameters obtained from circular dichroism, electron paramagnetic resonance and X-ray photoelectron spectroscopy (see also ref.1). There was also no detectable change in the electron absorption spectra compared to the untreated copper protein.

The search for a genuine biochemical function of superoxide dismutase (erythrocuprein) is continuously going on (1,2). While the exclusive reaction of superoxide dismutase with the monovalently charged oxygen radical is favoured even in cellular biochemistry (2), we presented some data indicating considerable doubts regarding the specificity of the enzyme catalysed superoxide dismutation (3-7). It has been shown by pulse radiolysis that Cu(II) alone was twice as active as the protein bound Cu(II) found in superoxide dismutase. Furthermore, the hydrophobic $Cu(tyr)_2$ complex survived the treatment of serum albumin, a naturally occurring chelator. The rate constant for superoxide dismutation in the presence of $Cu(tyr)_2$ was $1.0 \times 10^9\ M^{-1}\ s^{-1}$ compared to $1.3 \times 10^9\ M^{-1}\ s^{-1}$ calculated per equivalent of enzyme-bound Cu(II) (4,5). With the decay of CrO_8^{3-} in aqueous solution the complex nature of the reaction between a reducing agent and molecular oxygen was shown (6,7,9). To our great surprise native superoxide dismutase was capable to supress the singlet oxygen induced chemiluminescence in the absence of chemiluminescent agents in a very specific manner. In this system all model Cu-chelates were active less than 0.2% (7). Due to the complex behaviour of CrO_8^{3-} in aqueous solutions we considered photochemically generated singlet oxygen as a convenient source for this excited oxygen species. $^1\Delta_g O_2$ was produced by irradiating oxygen saturated ^2H_2O solutions in the presence of methylene blue (10-12). In order to prolong the lifetime of $^1\Delta_g O_2$ in water by at least one order of magnitude ^2H_2O was employed. Uncontrolled scavenging of singlet oxygen by histidine residues was avoided by irradiating the solutions for at least one hour prior to the addition of 1,3-diphenylisobenzofuran, a powerful and established singlet oxygen trapping agent (13-15). Using this system the reactivity of superoxide dismutase to supress singlet oxygen formation was examined and compared with the reactivity of other metal proteins including ceruloplasmin, hemocyanin, diamine oxidase, and some hemoproteins. Furthermore the inhibitory action of CN^-, N_3^-, EDTA and NH_3 was studied.

MATERIALS AND METHODS

All chemicals employed were of reagent grade quality or better. NaO^2H, $^2H_3PO_4$ and ^2H_2O Merck, Darmstadt. 1,3-diphenylisobenzofuran Aldrich, Milwaukee. Dibenzoylbenzene EGA-Chemie, Steinheim. Methylene blue Serva, Heidelberg. Hemocyanin and diamine oxidase Calbiochem, San Diego. Catalase, horseradish peroxidase and hemoglobin Boehringer, Mannheim. Superoxide dismutase was isolated from yeast following the procedures given in (16,17). Apo-superoxide dismutase and ceruloplasmin were a gift from Dr.H.-J. Hartmann, Physiol.-chem. Inst. Tübingen. $^1\Delta_g O_2$ was generated by irradiating an oxygen saturated solution of methylene blue in ^2H_2O using a tungsten light source (Osram 100 W, Krypton lamp). To avoid the direct oxidation of

1,3-diphenylisobenzofuran by light (18) only light above 600 nm was allowed to enter the cuvette. The assay was performed in a 23 mm light path quartz cell. The concentrations of the different components were: phosphate buffer 100 mM, p^2H 7.4, methylene blue 89 µM, 1,3-diphenylisobenzofuran 34 µM (added as a concentrated stock solution in 50 µl acetonitrile), copper chelates 36 µM. The total volume was 8.2 ml. The assay solution was irradiated 1 h before the reaction was started by adding 1,3-diphenylisobenzofuran. One ml aliquots were removed at zero time and after 5, 10, 20, 30 and 40 min. The formation of dibenzoylbenzene was monitored at 420 nm in a Unicam SP 1800 spectrophotometer.

For control 1,3-diphenylisobenzofuran and dibenzoylbenzene were detected using thin layer chromatography (19). Histidine was assayed following the method given by Jori et al. (20). EPR spectra were run on a Varian V-4502-11 spectrometer. The circular dichroism was measured on a JASCO 20 unit. X-ray photoelectron spectroscopy was performed on a Varian VIEE spectrometer equipped with an on line Varian 620L, 8K computer.

RESULTS

Due to the strong quenching effect of H_2O on $^1\Delta_gO_2$ which is roughly in the order of 10^5 M^{-1} s^{-1} (13) ^2H_2O was used instead. Indeed, the half-lifetime of the photochemically generated $^1\Delta_gO_2$ was high enough to detect a significant oxidation of 1,3-diphenylisobenzofuran. Dibenzoylbenzene was characterized by thin layer chromatography indicating the successful oxidation of 1,3-diphenylisobenzofuran by $^1\Delta_gO_2$. The uncontrolled inhibition of the oxidation process by histidine was avoided by irradiating the cuvette for 1 h prior to the start of the experiment. After this pretreatment neither histidine nor the histidine residues in the apoprotein could be chemically detected (see also 20).

Attributable to the high rate of 1,3-diphenylisobenzofuran oxidation with $^1\Delta_gO_2$ (0.8×10^9 M^{-1} s^{-1}) (13) at least the same Cu(II) concentration was added. In the presence of superoxide dismutase-Cu(II) a significant and rather specific inhibition of the 1,3-diphenylisobenzofuran oxidation can be seen (Fig. 1). No such inhibition was seen when apo-superoxide dismutase, serum albumin and some copper or iron proteins were employed (Table 1). The specificity was even more distinct when Cu(II) was added as

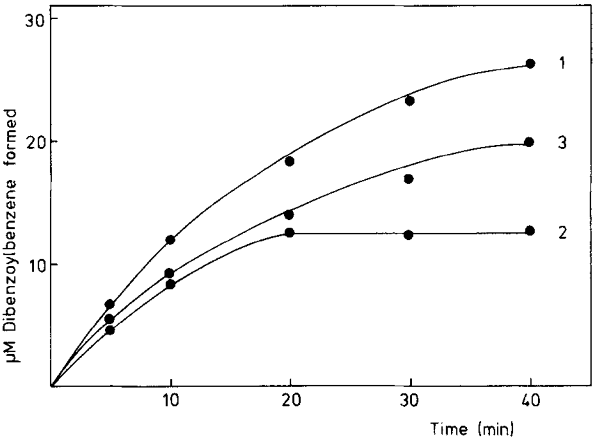

Figure 1: Time course of $^1\Delta_gO_2$ induced formation of dibenzoylbenzene in the presence and absence of superoxide dismutase. ① No addition, ② + 36 µM superoxide dismutase-Cu(II), ③ + 36 µM superoxide dismutase-Cu(II) + 40 µM cyanide. To minimize dilution effects all additions were made in less than 150 µl. 23 mm light path quartz cells were irradiated with a 100 W tungsten light source. Light filters were: a concentrated solution of methylorange and a Kodak 29 filter. After irradiation for 1 h prior to the start of the measurements the 1,3-diphenylisobenzofuran was added. Readings were taken from 1ml aliquots at 420 nm in a 10 mm light path glass cell.

$Cu(lys)_2$, $Cu(his)_2$, $Cu(tyr)_2$ or as the aquo complex. All these low molecular weight Cu-chelates could not successfully compete with the singlet oxygen trapping agent (Table 2). Apart from the unsuccessful Cu(II) catalysis the Cu(II) bound histidine was completely damaged as shown by chemical analysis before and after treatment with $^1\Delta_gO_2$.

The inhibitory action of some established metal chelators was investigated. Cyanide proved the most potent agent (Fig. 1) and was consistent with its reactivity known for other superoxide dismutase assays (1,2). Indeed, this would indicate that the copper ion is actually involved in the catalytic process. Azide proved to be almost as active (42% inhibition). The presence of EDTA or NH_3 caused no significant inhibition. A parallel could

Metal Protein	µMoles Dibenzoylbenzene formed x l^{-1}	% Inhibition
None	26.2 ± 1.0	0.0 ± 4
native superoxide dismutase	12.0 ± 0.6	54.0 ± 5
the apoprotein	21.4 ± 0.9	18.3 ± 4
Cu_4-serum albumin	23.1 ± 0.9	12.2 ± 4
serum albumin	23.1 ± 0.9	12.2 ± 4
hemocyanin	22.8 ± 0.9	13.0 ± 4
ceruloplasmin	22.8 ± 0.9	13.0 ± 4
diamine oxidase	23.1 ± 0.9	12.2 ± 4
horseradish peroxidase	22.8 ± 0.9	13.0 ± 4
hemoglobin	23.1 ± 0.9	12.2 ± 4
catalase	21.8 ± 0.9	16.8 ± 4

Table 1: Dibenzoylbenzene formation in the presence of different copper or iron proteins after 40 min. irradiation. The concentration of the respective protein bound metal ion was 36 µM. For further experimental details see caption to fig. 1.

be drawn to the superoxide dismutation where the last two complexing species were also unable to affect the enzymic activity of the cupreins.

The nativity of superoxide dismutase after $^1\Delta_gO_2$ treatment was ascertained by examining the chemical and physicochemical properties. At the end of the irradiation experiment superoxide dismutase was separated on a Sephadex G-25 column. One single homogeneous peak was monitored in the elution diagram. The chemical parameters were in accordance with those already described (20). All spectral properties including electronic absorption spectra, X-ray photoelectron spectroscopy, the electron paramagnetic resonance data and the circular dichroism remained

Cu-complex	µMoles Dibenzoylbenzene formed x 1^{-1}	% Inhibition
None	26.2 ± 1.0	0.0 ± 4
native superoxide dismutase	12.0 ± 0.6	54.0 ± 5
$Cu(tyr)_2$	24.8 ± 1.0	5.3 ± 4
$Cu(lys)_2$	25.3 ± 1.0	3.5 ± 4
$Cu(his)_2$	25.5 ± 1.0	2.7 ± 4
$Cu^{2+}aq$	26.0 ± 1.0	0.8 ± 4

Table 2: Dibenzoylbenzene formation in the presence of different Cu(II)-chelates after 40 min. irradiation. Values are calculated on an equivalent basis for Cu(II). Experimental details as in the legend to Fig. 1.

unchanged throughout. Regarding the enzymic function either assay (1,2,6,7) for superoxide dismutase activity showed no difference between the native and the irradiated superoxide dismutase.

To exclude possible oxidation of 1,3-diphenylisobenzofuran by O_2^- solid KO_2, 1 mM solutions of KO_2 in dimethylsulphoxide and electrolytically prepared O_2^- were added to the assay mixture in the absence of superoxide dismutase. No oxidation of 1,3-diphenylisobenzofuran was detected. Enzymically generated O_2^- using the xanthine / xanthine oxidase system did not affect the $^1\Delta_g O_2$ trapping agent. These observations support very strongly the complete absence of superoxide.

DISCUSSION

As in the case of the CrO_8^{3-} decay in aqueous solutions where a significant and specific reaction of superoxide dismutase was shown, the same specificity can be seen in the photochemically generated $^1\Delta_g O_2$ system. In fact, the unique reactivity of superoxide dismutase is even more pronounced in ^2H_2O. It could be argued why using ^2H_2O , this is not a natural system? We do not want to say that pulse radiolytic studies are of biological relevance. However, it should be kept in mind that biochemistry in the living cell is not exclusively restricted to aqueous systems. The solid phase chemistry, for example, of the alkaline earth metal phosphates and carbonates as well as the reactions in solvents of low polarity including lipids and membranes or in the hydrophobic regions of proteins and polynucleotides should be pointed out. It is certainly correct that we do not really need neither a superoxide dismutase nor a "singlet oxygen decontaminase" in aqueous systems. Both oxygen species will be rapidly scavenged by H_2O which is present in concentrations up to 55 M compared to the µM amounts of superoxide dismutase. However, a completely different situation has to be considered during oxidative processes where lipids, macromolecular lipid bilayers of membranes or the above cited hydrophobic regions presented in many macromolecules are involved. In these systems the half-lifetime of singlet oxygen will be substantially increased and the deleterious action of these excited oxygen species may proceed rather uninhibited unless there are scavenging enzymes available.

It has been shown that superoxide dismutase is of a remarkable resistance versus $^1\Delta_gO_2$. An attractive conclusion might be brought forward. The 22 kcal of $^1\Delta_gO_2$ above the triplet ground state oxygen will be accepted by the protein portion of superoxide dismutase. The enzyme itself will be in an excited state and the energy finally given away to adjacent H_2O molecules. Throughout this process both the metal and the protein conformation play a major role. An appropriate tertiary structure is essential to fulfill all the requirements of singlet

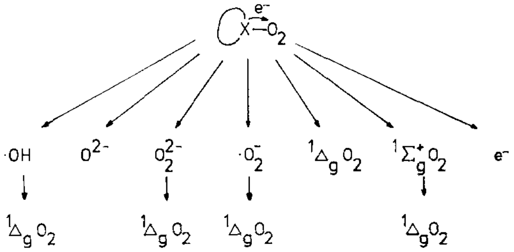

Figure 2: Proposed decay of a XO_2 complex

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oxygen decontamination.

Looking at the reaction between oxygen and a reducing component it can be envisaged that all sorts of excited oxygen species may be generated depending on the nature of the surrounding macromolecular portion (Fig. 2). The different pathways of oxygen decay are dictated by the respective macromolecular ligand. In this system superoxide is just one special case where singlet oxygen could be formed. The conclusion can be drawn that superoxide dismutase prevents the deleterious action of singlet oxygen. It may react with XO_2 complexes or singlet oxygen directly. This latter question must be answered by separate studies on a molecular level. The exclusive superoxide dismutation in biological systems, however, cannot be regarded as the predominant role of this copper protein.

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