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Singlet oxygen decontaminating activity of erythrocuprein (superoxide dismutase)

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SUMMARY

Erythrocuprein is both a most powerful and specific metalloprotein able to decontaminate highly reactive $^1\Delta_g$ singlet oxygen being exclusively generated by K_3CrO_8. Even in concentrations up to $2 \cdot 10^{-10}$ M erythrocuprein displayed a clearly detectable singlet oxygen decontaminating activity. In contrast to the considerable superoxide dismutase activity of low molecular weight copper chelates (4%) the singlet oxygen decontaminating activity of these model compounds was remarkably low by 4 orders of magnitude compared to the respective activity of native erythrocuprein. From these data it must be concluded that in aerobic biological systems erythrocuprein is acting as a singlet oxygen decontaminating enzyme rather than as a superoxide dismutase. This conclusion was made in the light of the well established specificity of the enzyme and the deleterious action of ubiquitous singlet type oxygen species even in the absence of superoxide.

The long known copper protein called erythrocuprein was originally discovered by Mann and Keilin in 1939 [1]. The cupreins were found to contain 2 gatoms of copper and zinc each[2] and to act as superoxide dismutases[3]. The evaluation of the rate constant of the enzyme catalyzed disproportionation of superoxide yielded a numerical value of approximately $2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$. The same constant was measured throughout the pH range from pH 4 to 9 (refs 4–7). However, the central question regarding the physiological function of the cupreins is still not fully understood.

In the laboratories of Rotilio *et al.*[8] and Weser and co-workers[9–11] the main

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.

physiological role of erythrocuprein was proposed that it scavenged highly reactive species of singlet oxygen. The decontamination of these powerful singlet oxygen species giving 22 kcal ($^1\Delta_gO_2$) or even 37 kcal ($^1\Sigma_g^+O_2$) would be beneficial for most aerobic metabolic processes[12,13]. In the absence of an appropriate singlet oxygen scavenger all kinds of unsaturated compounds or reducing agents of biological significance would be rapidly oxidized by these harmful oxygen species.

For example, it was shown that $^1\Sigma_g^+O_2$ can be generated from potassium superoxide[14] or during photochemical reactions[12]. Unfortunately, the unequivocal answer as to whether or not singlet oxygen can be formed directly during a chemical reaction in the dark, avoiding its superoxide-mediated formation, was not possible[8-11,15-17]. This was due to the complexities of the assay mixtures employed. With the report by Peters *et al.*[18] final evidence was presented that $^1\Delta_gO_2$ was exclusively formed in the dark using an aqueous solution of CrO_8^{3-} at neutral pH.

$$4\,CrO_8^{3-} + 2\,H_2O = 4\,CrO_4^{2-} + 7\,O_2 + 4\,OH^-$$

There was also strong indication that no superoxide at all was generated during this chemical reaction. Thus, this result supports earlier observations of a direct singlet oxygen generation in biological systems avoiding the pathway *via* superoxide. In the present study we wish to report that erythrocuprein is both a most powerful and specific metalloprotein to decontaminate highly reactive $^1\Delta_gO_2$ generated by K_3CrO_8.

Cuprein was isolated from bovine erythrocuprein using the methods given in refs 5 and 13. Crystalline K_3CrO_8 was prepared by treating an ice cold alkaline CrO_4^{2-} solution with H_2O_2 (ref. 19). The copper chelates were prepared as described elsewhere[10]. Bovine cytochrome *c* and catalase were from Boehringer, Mannheim; KO_2 from K and K laboratories, Hollywood; *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES) buffer and dimethylsulphoxide from Merck, Darmstadt; luminol from Roth, Karlsruhe.

Singlet oxygen destruction assay: During the aqueous decomposition of CrO_8^{3-} $^1\Delta_gO_2$ was gradually formed[18]. The instantaneous light emission from $^1\Delta_gO_2$ at 1270 nm was shifted with luminol into the visible region and was monitored employing a Packard scintillation counter model 2002. The reaction was started by adding the weighed amount of solid crystalline K_3CrO_8 to the assay mixture during intensive agitation of the scintillation vial. The measurements were carried out for 12 s. An interval of 6 s was allowed for printing the numerical values. Counting was performed in the dark at room temperature. The setting was exactly as for ^3H counting and the coincidence circuit was turned on. The assay mixture was composed of: HEPES buffer, 0.1M, pH 7.8; luminol, 0.3 mM; K_3CrO_8, 0.3 mM; the total volume was 5.1 ml. The chemiluminescence curve was integrated and the total counts were compared with those obtained after the addition of erythrocuprein or the Cu^{2+} chelates.

Superoxide dismutase assay was performed using the cytochrome *c* reductase test[10] which was modified employing potassium superoxide in dimethylsulphoxide. The Cu^{2+} concentration of different Cu^{2+} chelates and erythrocuprein required to yield 50% inhibition of the cytochrome *c* reduction was determined.

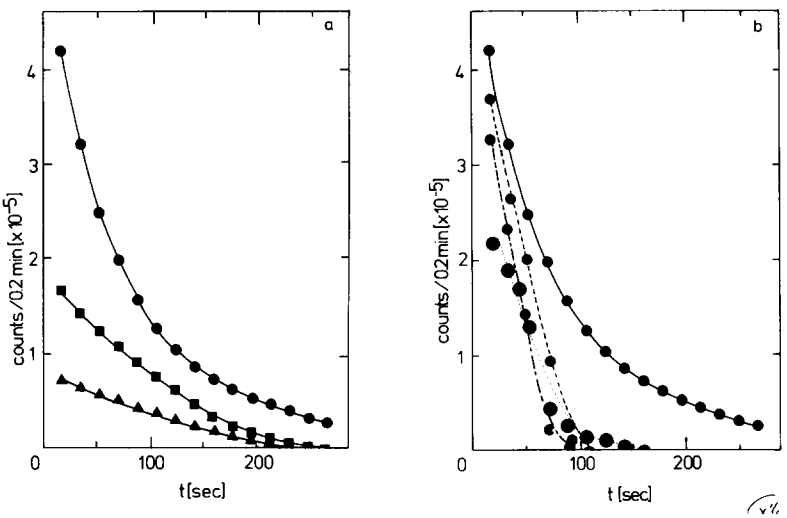

Fig. 1. (a) $^1\Delta_gO_2$ decay mediated by luminol in the presence of erythrocuprein. Erythrocuprein concentrations: ■——■, 2.4 nM; ▲——▲, 12 nM; ●——●, omitted. The first count was performed 20 s after the addition of solid K_3CrO_8. During the gradual aqueous decomposition of CrO_8^{3-} into $^1\Delta_gO_2$ the light emission from Δ_gO_2 detectable at 1270 nm is very fast[18]. This light emission was shifted into the visible region employing luminol. The assay mixture contained: 0.1 M HEPES buffer, pH 7.8; luminol, 0.3 mM; K_3CrO_8, 0.3 mM; the total volume was 5.1 ml. Counting was performed in polypropylene vials (20 ml) using a Packard scintillation counter. All measurements were taken in the dark at room temperature. The chemiluminescence curve was integrated and the total counts compared in the different experiments. (b) Δ_gO_2 decay mediated by luminol in the presence of copper chelates. ——, control as above; . . . . , $CuSO_4$, 10 000 nM; - - - - , $Cu(Lys)_2$, 21 000 nM; -.-.-.-. , $Cu(His)_2$, 21 000 nM. Experimental details as in a.

Crystalline K_3CrO_8 proved a most convenient reagent producing $^1\Delta_g$ oxygen at neutral pH. Concentrations of about 10^{-4} M did not affect the protein and yielded an extraordinarily high counting rate of approx. 10^5 cps at zero time (Fig. 1). The light emission continued for quite a long time and was measurable up to 6 min. In the presence of erythrocuprein the chemiluminescence was significantly diminished (Fig. 1a). A slight scavenging of singlet type oxygen was detectable already at erythrocuprein concentrations of about 10^{-10} M. In contrast to the native enzyme, much higher concentrations of low molecular weight copper chelates or the Cu^{2+}. aq ion were required to cause the same reactivity (Fig. 1b). Concentrations of copper chelates ranging about 10^{-5} M (Table II) were required to inhibit the chemiluminescence by 50%.

The proportionality of the erythrocuprein-inhibited chemiluminescence was examined and yielded a linear calibration curve (Fig. 2). The erythrocuprein concentration required to produce a 50% inhibition of the integrated chemiluminescence was taken as an enzyme unit for quantitating the singlet oxygen decontaminating activity.

In Tables I and II the superoxide dismutase activity and the singlet oxygen decontaminating activity of native erythrocuprein and different copper chelates were

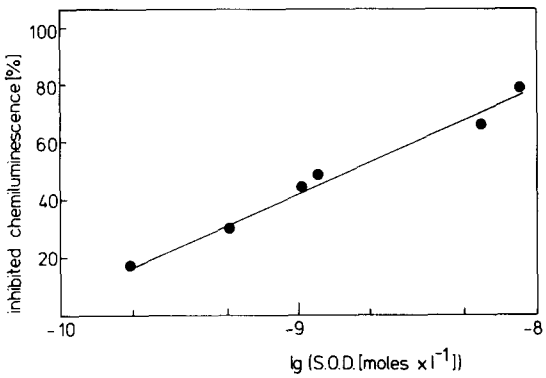

Fig.2. Proportionality of the erythrocuprein inhibited chemiluminescence induced by $^1\Delta_g O_2$. S.O.D., singlet oxygen decontaminating activity.

TABLE I

SUPEROXIDE DISMUTASE ACTIVITY OF DIFFERENT COPPER CHELATES IN THE KO_2 ASSAY
The assay mixture contained: Glycine buffer, 0.1 M, pH 9.2; cytochrome c_{ox}, 56 μM; catalase, 2.8 I.U.; total volume 1.5 ml. The reaction was started with 0.1 ml of a nonaqueous (dimethyl sulphoxide) solution containing 1.1 mM KO_2. Readings were taken in an Unicam SP 1800 at 550 nm, 20 °C. For further details see refs 10 and 13.

Cu^{2+} chelate	*Required equivalent of chelated copper to yield 50% inhibition of cytochrome c reduction Cu^{2+} (moles·l^{-1}·10^6)*
Native erythrocuprein	0.086
Boiled erythrocuprein	9.2
Apoerythrocuprein	6.4
Cu–EDTA	96
$Cu(His)_2$	2.0
$Cu(Lys)_2$	5.7

compared. The earlier observation of the superoxide dismutase activity of $Cu(His)_2$ and $Cu(Lys)_2$ (ref. 10) was confirmed using non-enzymically produced superoxide by adding potassium superoxide dissolved in dimethylsulphoxide to an aqueous cytochrome c_{ox} solution. In general, the superoxide dismutase activity of native cuprein and the model chelates was not too much different. The difference was only about 2 orders of magnitude for most (Cu–EDTA excepted).

This was contrasted by the singlet oxygen decontaminating activity of the copper compounds studied (Table II). The reactivity of native erythrocuprein was surprisingly specific. The model copper chelates and $CuSO_4$ were virtually inactive compared to the native enzyme. No significant quenching of the chemiluminescence was observed within the first 30–40 s. In comparing the fully integrated scintillation curves the difference was

TABLE II

SINGLET OXYGEN DECONTAMINATING ACTIVITY OF COPPER CHELATES AND ERYTHROCUPREIN

For experimental details see Fig. 1a. The evaluation was carried out as depicted in Fig. 2.

Cu^{2+} chelate	*Required equivalent of chelated copper to yield 50% inhibition of $^1\Delta_gO_2$ induced chemiluminescence Cu^{2+} (moles·l^{-1}·10^6)*	*Specific activity (units·$mole^{-1}$·10^{-5})*
Native erythrocuprein	0.0036	2800
Boiled erythrocuprein	260	0.038
Apoerythrocuprein	0.42	24
Cu–EDTA	300	0.033
$Cu(His)_2$	10	1
$Cu(Lys)_2$	23	0.43

4 orders of magnitude which implies a much higher specificity for this enzymic reaction of the cupreins. The powerful reactivity of erythrocuprein is further demonstrated by the fact that the apoprotein displayed a detectable enzymic activity due to traces of copper which were undetectable by atomic absorption measurements or EPR spectroscopy. No such difference between apoprotein and the boiled native enzyme was observed using the superoxide dismutase assay.

The evidence that during the reaction of CrO_8^{3-} with water $^1\Delta_gO_2$ is exclusively generated[18] prompted us to use this chromium peroxicomplex as a substrate for erythrocuprein. Stomberg and Brosset[21] showed in 1960 that the chromium ion is in the oxidation state +5 and surrounded by four O_2^{2-} groups. So far no evidence from X-ray crystallographic data has been obtained for the presence of superoxide. The possibility that O_2^- may be formed during the aqueous decomposition of CrO_8^{3-} was examined using the cytochrome *c* reductase assay[10] and the nitro blue tetrazolium staining method[20]. Both assays for superoxide proved absolutely negative. CrO_4^{2-} and CrO_8^{3-} did not disturb these assays since additional potassium superoxide caused a strong positive reaction for the presence of O_2^-.

Using CrO_8^{3-} as a substrate we were able to demonstrate the specificity and efficiency of the cupreins for scavenging highly energetic $^1\Delta_gO_2$. The long known phenomenon that singlet oxygen species are evolved during biochemical reactions led to the assumption that these oxygen species were the actual physiological substrates for erythrocuprein[8–10,12,13]. However, the question remained open concerning the superoxide-mediated formation of these oxygen species. No final decision could be made due to the rather complex nature of the assay systems employed. With the present study unequivocal evidence has been obtained that superoxide is not involved in the singlet oxygen production.

The high specificity of erythrocuprein with regard to scavenging singlet oxygen supports the statement of the entatic state of metal ions in metalloenzymes[22,23]. The protein portion of the native erythrocuprein provides the proper ligand for the metal ions

producing this entatic situation. In contrast, the low molecular weight copper chelates are virtually inactive because the copper would not be in this entatic state required to induce this singlet oxygen decontaminating activity.

Considering all the presented data the conclusion must be made that the main physiological function of the cupreins is the scavenging of singlet oxygen species rather than catalyzing the superoxide disproportionation. The occurrence of superoxide would be just one specific pathway for singlet oxygen formation.

The protective action of a singlet oxygen decontaminating enzyme upon membrane lipids, all sorts of unsaturated compounds and a great number of reducing agents can be considered essential in the metabolism of the aerobic cell.

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REFERENCES

1 Mann, T. and Keilin, D. (1939) *Proc. R. Soc. London, Ser. B* 126, 303–315
2 Carrico, R.J. and Deutsch, H.F. (1970) *J. Biol. Chem.* 245, 723–727
3 McCord, J.M. and Fridovich, I. (1969) *J. Biol. Chem.* 244, 6049–6055
4 Rotilio, G., Bray, R.C. and Fielden, M.E. (1972) *Biochim. Biophys. Acta* 268, 605–609
5 Klug, D., Rabani, J. and Fridovich, I. (1972) *J. Biol. Chem.* 247, 4839–4842
6 Klug, D., Fridovich, I. and Rabani, J. (1973) *J. Am. Chem. Soc.* 95, 2786–2790
7 Bannister, J.V., Bannister, W.H., Bray, R.C., Fielden, E.M., Roberts, P.B. and Rotilio, G. (1973) *FEBS Lett.* 32, 303–306
8 Finazzi-Agro, A., Giovagnoli, C., del Sole, P., Calabrese, L., Rotilio, G. and Mondovi, B. (1972) *FEBS Lett.* 21, 183–185
9 Weser, U. and Paschen, W. (1972) *FEBS Lett.* 27, 248–250
10 Joester, K.E., Jung, G., Weber, U. and Weser, U. (1972) *FEBS Lett.* 25, 25–28
11 Zimmermann, R., Flohé, L., Weser, U. and Hartmann, H.J. (1973) *FEBS Lett.* 29, 117–120
12 Politzer, I.R., Griffin, G.W. and Laseter, J.L. (1971) *Chem. Biol Interact.* 3, 73–93
13 Weser, U. (1973) *Structure and Bonding* 17, in the press
14 Khan, A.U. (1970) *Science* 168, 476–477
15 Chan, H.W.S: (1971) *J. Am. Chem. Soc.* 93, 2357–2358
16 Goda, K., Chu, I.W., Kimura, T. and Schaap, A.P. (1973) *Biochem. Biophys. Res. Commun.* 52, 1300–1306
17 Pederson, T.C. and Aust, S.D. (1973) *Biochem. Biophys. Res. Commun.* 52, 1071–1078
18 Peters, J.W., Pitts, Jr, J.N., Rosenthal, I. and Fuhr, H. (1972) *J. Am. Chem. Soc.* 94, 4348–4350
19 Riesenfeld, E.H., Wohlers, H.E. and Kutsch, W.A. (1905) *Chem. Ber.* 38, 1885–1898
20 Beauchamp, C. and Fridovich, I. (1971) *Anal. Biochem.* 44, 276–287
21 Stomberg, R. and Brosset, C. (1960) *Acta Chem. Scand.* 14, 441–452
22 Vallee, B.L. and Williams, R.J.P. (1968) *Proc. Natl. Acad. Sci. U.S.* 59, 498–505
23 Behnke, D. and Vallee, B.L. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 2442–2445