SUPEROXIDE DISMUTASE ACTIVITY OF LOW MOLECULAR WEIGHT Cu^{2+}-CHELATES STUDIED BY PULSE RADIOLYSIS

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1. Introduction

The discovery of an accelerated superoxide dismutation [1] using erythrocuprein led to an overwhelming number of studies on this metalloprotein (for a review see ref. [2]). The mechanism of this enzymic catalysed reaction was successfully studied by pulse radiolysis [3–5]. It was intriguing to realize that erythrocuprein which has apparently evolved [6] to perform so simple a reaction proved, in some ways, far from simple. The situation turned out even more complicated with the knowledge that free Cu^{2+} catalyses superoxide dismutation in acidic media in a much faster way compared to the reactivity of erythrocuprein under physiological pH conditions [7]. Furthermore, it was demonstrated that low molecular weight copper chelates display marked superoxide dismutase activity at physiological pH values employing the cytochrome *c* reductase assay [8]. In this context it was of high importance to measure the rate constants for reaction between some of these cupric-peptide chelates and the superoxide ion generated by pulse radiolysis at pH 7.5.

The reaction of chelated Cu^{2+} with superoxide was very fast throughout. The respective second order rate constants of either equivalent of Cu^{2+} ranged from 0.6×10^9 M^{-1} sec^{-1} ($Cu(lys)_2$) up to 1.3×10^9 M^{-1} sec^{-1} (erythrocuprein). The free Cu^{2+} aq complex was even more active (2.7×10^9 M^{-1} sec^{-1}). However, in the presence of bovine serum albumin, a naturally occurring chelating agent, the reactivity of Cu^{2+} aq was clearly diminished. This was not the case using the Cu (gly–his)$_2$ complex. This result supports earlier conclusions that superoxide dismutase activity is not restricted to erythrocuprein. Appropriate Cu^{2+}-complexes are capable of the same reactivity provided those coordination sites required for the binding of O_2^- are not blocked by other chelators.

2. Materials and methods

Crystalline Cu^{2+}-chelates were prepared in a way similar to the method given in [8,9]. The stoichiometry was 1 Cu per 2 moles of ligand as confirmed by elementary analysis. All commercial reagents employed were of analytical grade purity or better. Water was triply distilled and pyrrolixed. Gly–his was from Cyclo Chemical, Los Angeles, USA. Gly–his–leu was a generous gift from Dr U. Weber, Physio.-chem. Institut, Tübingen. Cuprein was prepared from bovine erythrocytes as published elsewhere [2].

Superoxide assay: A Febretron pulse radiolysis unit equipped with a suitable optical detection system (Zeiss PMQ II monochromator, EMI 9258 photomultiplier, Tectronix 7704 oscilloscope) was employed. The light source was a xenon lamp. O_2^- radicals were

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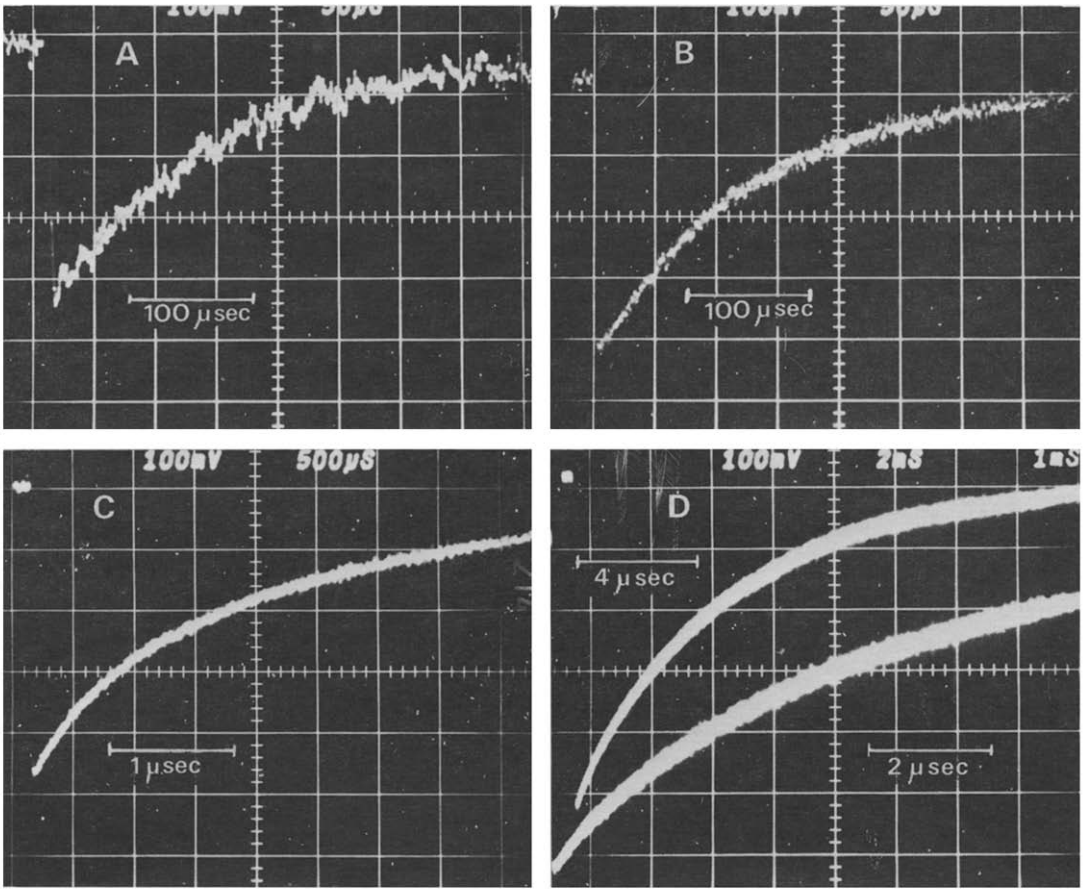

Fig. 1. Oscilloscope traces showing the decay of superoxide in the presence of A) erythrocuprein; B) $Cu(lys)_2$; C) $CU(gly-his)_2$; D) $Cu(gly-his-leu)_2$. The 2 cm cells were saturated with oxygen and contained 1 mM sodium formate. The pH was 7.8 adjusted with NaOH. O_2^- was produced by a 1.81 MeV electron pulse of 40 nsec duration. The initial O_2^- concentration was 35 ± 10 μM. The O_2^- decay was monitored at 245 nm using a Tectronix 7704 oscilloscope combined with a Polaroid camera. A Febetron pulse radiolysis unit was employed throughout. A6–15 μM erythrocuprein, sweep rate 50 μsec/cm; B – 10 μM $Cu(gly-his)_2$, sweep rate 500 μsec/cm; C – 1 μM $Cu(gly-his)_2$, sweep rate 500 μsec/cm; D – 1 μM $Cu(gly-his-leu)_2$, sweep rate upper trace 2 msec/cm, lower trace 1 msec/cm. No difference was seen employing Cu^{2+} alone.

produced by irradiation of O_2-saturated aqueous solutions in 2 cm cells by a pulse of 1.81 MeV electrons of 40 nsec duration. 1 mM sodium formate was added to scavenge OH radicals and to duplicate the O_2^- yield. The decay of superoxide was measured at 245 nm where it showed maximal absorption. The total O_2^- concentration was calculated from the radiation dose. The rate constants were determined as in [10] using a Wang 2200 computer.

3. Results

Superoxide was generated by a 40 nsec pulse using oxygen saturated aqueous solutions. The O_2^- concentration ranged in the other of 40 μM. The decay of O_2^- followed first order (fig. 1). The rate constants for reaction between different Cu^{2+}-chelates and superoxide was obtained by plotting log A versus time (fig. 2). It was intriguing to see the essentially same character of the O_2^- decay in the presence of either copper chelate. Erythrocuprein made no exception and showed the characteristics of the common reactivity of cupric ions with the monovalently charged oxygen species.

The numerical values of the second order rate

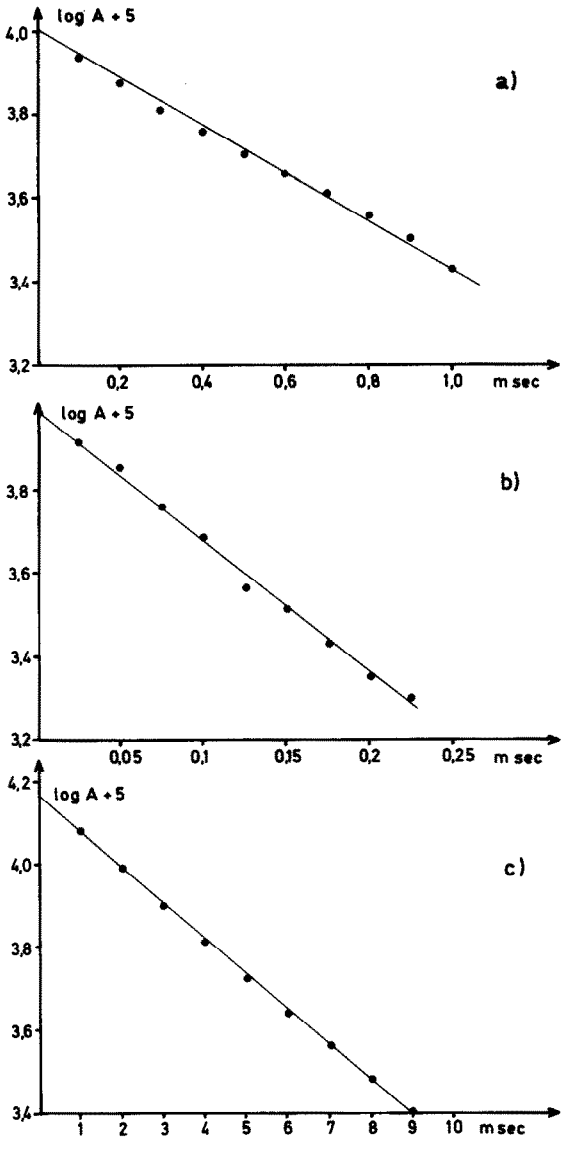

Fig. 2. First order plots using a) 1 μM Cu^{2+} aq, b) 5 μM erythrocuprein, c) 1 μM Cu(gly–his–leu)$_2$. The data were taken from the respective photographs of the oscilloscopic traced O_2^- decay. The corresponding measurements of Cu(lys)$_2$ and Cu(gly–his)$_2$ gave also strictly linear first order plots.

constants are compared in table 1. The cuprio–aquo complex displayed the fastest rate constant followed by the reactivity of the copper of erythrocuprein. For comparative reasons especially with regard to the

Table 1
Second order rate constants of chelated Cu^{2+} expressed per equivalent of copper

Cu-complex	Cu^{2+}-concentration (μM)	$k_{245} \times 10^{-9}$ M^{-1} sec^{-1}
Cu^{2+} aq	1.0	2.70 ± 0.20
Erythrocuprein	1.0	1.30 ± 0.10
	5.0	1.30 ± 0.10
Cu(lys)$_2$	1.0	0.56 ± 0.10
	10.0	0.60 ± 0.10
Cu(gly–his)$_2$	1.0	0.29 ± 0.02
	10.0	0.31 ± 0.02
Cu(gly–his–leu)$_2$	1.0	0.21 ± 0.02

The numerical values were calculated by dividing the first order constants of fig. 2 by the employed Cu^{2+} concentration. For further experimental details see legend to fig. 1.

other Cu^{2+}-chelates all constants were expressed per equivalent of copper. The numerical value for the whole erythrocuprein molecule (2.6×10^9 M^{-1} sec^{-1}) was in agreement with the corresponding data obtained in other laboratories. [4,5]. To our great surprise the rate constants of the copper chelates using lysine and some histidine containing peptides were within the same order of magnitude as found for the respective copper constants in erythrocuprein.

It was demonstrated earlier [8] that the superoxide dismutase activity of the free Cu^{2+} aq complex can be markedly inhibited in the presence of chelating agents. This aspect was also examined using a polymer chelator of biological significance. The addition of bovine serum albumin, indeed, resulted in a clearly detectable slower rate constant (table 2). No such inhibition was observed when the Cu^{2+} ion was coordinated to gly–his prior to the pulse radiolysis assay.

Table 2
Second order rate constants of Cu^{2+} and Cu(gly–his)$_2$ in the presence and absence of bovine serum albumin (0.11 mg/mg)

Cu-complex (conc. 1 μM)	$k_{245} \times 10^{-9}$ M^{-1} sec^{-1} (without serumalbumin)	$k_{245} \times 10^{-9}$ M^{-1} sec^{-1} (with serumalbumin)
Cu^{2+} aq	2.70 ± 0.20	0.25 ± 0.05
Cu(gly–his)$_2$	0.29 ± 0.02	0.15 ± 0.05

Experimental details as in fig. 1 and table 1.

4. Discussion

The catalysis of superoxide dismutation by the cupric ion appears to be a widely distributed phenomenon of this, and perhaps a considerable number of other transition elements. For the successful catalysis in biological systems several requirements must be fulfilled. Due to extraordinary high rate constant of Cu^{2+}-mediated superoxide dismutation a fast exchange of coordinated water [11] is essential to allow the binding of the oxygen radical. This condition is well furnished by the free Cu^{2+} aq complex in strictly aqueous medium, but not in the presence of competing chelating agents. In the latter case the consequence would be undesired and unspecific binding with those copper coordination sites normally being essential for the reaction with superoxide. The protection of the active coordination sites was thought appropriate by selecting suitable chelating agents which would be in a position to hold these criteria. For a long time this privilege seemed possibly only for the copper ions in erythrocuprein [1] and according to recent studies studies should also be considered for the non blue copper protein galactose oxidase [12,13]. However, as we have shown some Cu^{2+} chelates employing simple amino acids and peptides proved capable to react with superoxide even in the presence of the naturally occurring chelator bovine serum albumin. In light of these results, therefore, it appears strongly advisable to devote substantial efforts on the genuine biochemical specificity of erythrocuprein a metalloprotein whose biological occurrence has been known for 35 years [14].

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