SUPEROXIDE DISMUTASE ACTIVITY OF Cu^{2+}–AMINO ACID CHELATES

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1. Introduction

The disproportionation of $O_2^- \cdot$ into H_2O_2 and O_2 was found enzymically catalysed by erythrocuprein [1–4]. Due to this reactivity the long known copper protein [5] was renamed superoxide dismutase [1]. Detailed enzymic studies [6, 7] led to the proposal of another important function of erythrocuprein, namely the scavenging of singlet oxygen in many oxidation reactions. At the moment no decision can be made which of these enzymic functions will be given privilege in metabolism. Before a final designation of this Cu-protein is suggested further studies regarding the specificity of the above mentioned biochemical reactions ought to be carried out.

We thought it worthwhile to examine the question as to which degree copper chelates using both the free amino acids or some low molecular weight peptides would be able to display similar enzymic activities. All amino acids being present in bovine erythrocuprein [8] were employed. The Cu^{2+}-complexes of lysine, histidine, tyrosine or some di- and tripeptides thereof displayed considerable biochemical activities.

The superoxide dismutase activity of both the native erythrocuprein and different Cu^{2+}–amino acid complexes were compared on a molar basis of chelated Cu^{2+}. In the cytochrome *c* reductase assay [1,4] enzymic activities of up to 5% $Cu(Lys)_2$ compared to native erythrocuprein were determined. Slightly higher activities were observed using the chemiluminescence test [6, 7]. In this alternative test $Cu(Lys)_2$ displayed 7.5% enzymic activity. As in the cytochrome *c* reductase assay only the Cu^{2+}–chelates of Tyr, His and Lys were enzymically active. Copper complexes of the other 14 amino acids normally being found in erythrocuprein were completely inactive.

2. Materials and methods

The homogeneity of all amino acids and peptides was examined employing thin-layer chromatography. Cu^{2+}–chelates were prepared at 40° in a way similar to the method given in [9] using CuO and the ligands. Erythrocuprein was prepared from freshly harvested bovine erythrocytes using organic solvents, DEAE 23 and gel chromatography [10]. The native Cu–protein was converted into the apoprotein on chelator equilibrated Sephadex columns as described elsewhere [11, 12]. Superoxide dismutase activity was assayed using the xanthine, xanthine oxidase reaction [4]. Alternatively the scavenging of singlet oxygen was measured employing the chemiluminescence test described by Arneson [6] and Finazzi Agro' et al. [7]. Chemiluminescence was measured in a Packard scintillation counter Model 2002 at room temp. The setting at the scintillation counter was exactly as used for [^{3}H] counting. Xanthine, xanthine oxidase, catalase and beef heart cytochrome c_{ox} were purchased from Boehringer, Mannheim. HEPES buffer and luminol were from Merck, Darmstadt.

3. Results

The ability to supress the xanthine–xanthine oxidase induced cytochrome *c* reductase activity was

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examined in the presence of erythrocuprein and different cupric chelates using the free amino acid residues and some low molecular weight peptides. In the presence of the metal-free ligands and Cu^{2+} alone no superoxide dismutase activity at all was determined. A marked activity was found if $Cu(Lys)_2$, $Cu(His)_2$ or CuTyr was used. Even some of the Cu^{2+}–peptides were considerably active (table 1).

Upon examining the quenching of singlet oxygen in the presence of the above cupric chelates a rather similar picture is obtained (fig. 1). About 4.8 nM of native erythrocuprein was necessary to inhibit the chemiluminescence by 50%. The superoxide dismutase of both the native enzyme and different Cu^{2+}–chelates were compared on a molar basis of chelated Cu^{2+} required to quench the chemiluminescence by 50%. According to this evaluation method the superoxide dismutase activities of the following Cu^{2+}–chelates were: $Cu(His)_2$, 4.9%; CuTyr, 5.6%; $Cu(Lys)_2$, 7.5%; Cu–Leu–Tyr, 4.8% and Cu–His–Leu–Gly, 6.4%. The free amino acids or peptides and Cu^{2+}·aq. in concentrations similar to the corresponding Cu^{2+}–complexes displayed no enzymic activity et all.

An interesting phenomenon was observed when the chemiluminescence assay was performed at pH 8.6 (fig. 2). The overall chemiluminescence was higher by one order of magnitude. At the beginning the cpm values were progressively diminished. Surprisingly, the light emission increased after 25 sec and a maximum was reached after 40 sec. In the presence of erythrocuprein or some Cu^{2+}–amino acid chelates the initial light emission was drastically reduced and the maximum was shifted towards a

Table 1
Superoxide dismutase activity of different Cu^{2+}–amino acid chelates.

Cu^{2+}-Chelate	Required equivalent of chelated Cu^{2+} to yield $[Cyt\ c_{red}] \times [Cyt\ c_{ox}]^{-1} = 1$ ($\mu moles^{-1} \times litre$)
Native bovine erythrocuprein	16.7
1-Cu–apoerythrocuprein	1.0
$Cu(Lys)_2$	0.9
CuTyr	0.6
$Cu(His)_2$	0.5
$Cu(His\text{-}methylester)_2$	0.2
Cu–Leu–Tyr	0.5
Cu–Lys–Ala	0.2
Cu–His–Leu–Gly	0.6
Cu–Gly–His–Leu	0
Cu–His–Leu–Leu	0.3
Cu–Leu–His–Leu	0.04
Cu–Leu–Leu–His	0
Cu^{2+}·aq	0
Cu–EDTA	0
Cu–bovine serum albumin	0

All the employed amino acids and peptides were in the L-form. The cytochrome *c* reductase assay was performed at 25° in a volume of 0.76 ml using 1 cm light path optical glass cells. The assay mixture contained: HEPES buffer, 50 mM, pH 7.8; xanthine, 0.33 mM; cytochrome c_{ox}, 27 μM; catalse, 320 I.U.: start with xanthine oxidase, 0.02 units (as defined in [19]) in 50 μl. The time course of the absorption at 550 nm was recorded in a Unicam SP 1800.

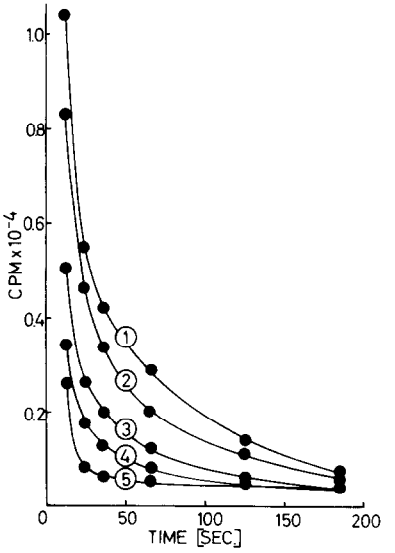

Fig. 1. Chemiluminescence assay of bovine erythrocuprein and different Cu^{2+}–amino acid complexes at pH 7.8. (1) None; (2) $Cu(Lys)_2$, 50 nM; (3) $Cu(His)_2$, 100 nM; (4) Cu–Tyr, 145 nM; (5) bovine erythrocuprein, 8 nM. The assay components were pipetted in a disposable scintillation vial at room temp. The total volume was 2.22 ml. The assay mixture was composed of: HEPES buffer, 50 mM; xanthine, 0.33 mM; catalase, 800 I.U; luminol, 1 mM; the reaction was started with 0.08 units (definition as in [19]) of xanthine oxidase after one min. The first reading was performed after 10 sec. During the counting the coincidence was turned on. The background was 4 ± 1 cpm.

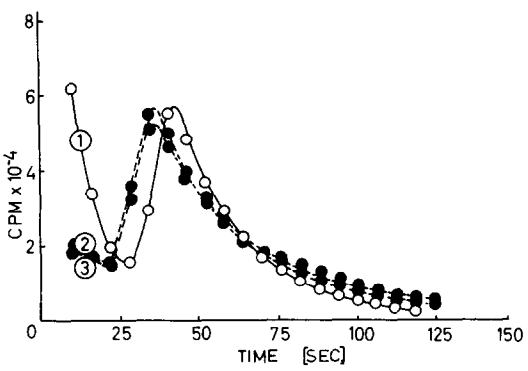

Fig. 2. Chemiluminescence assay of bovine erythrocuprein and $Cu(His)_2$ at pH 8.6. (1) None; (2) $Cu(His)_2$, 80 nM; (3) erythrocuprein, 0.3 nM. Further test conditions as in fig. 1. Note the increase of the light emission!

shorter time. There were no detectable differences of the relative enzymic activities at either pH value [18]. This was especially intriguing since the time course and the yield of the light emission were considerably different.

4. Discussion

Oxidase and catalase activities of low molecular weight cupric chelates have been reported by Pecht and Anbar [13] and Sigel [14]. Due to the present study, the superoxide dismutase activity of some Cu^{2+}–amino acid chelates has to be added to the biochemical reactivity of copper. This superoxide dismutase activity as determined by two different assay procedures is certainly not an artefact since the concentration of the employed Cu^{2+}–amino acids and Cu^{2+}–peptides were in the μmolar region. In many biochemical systems metal ion activation is usually observed in the presence of millimolar concentrations of metal ions [15]. Thus our observation can be considered as a rather specific biochemical reactivity of $Cu(Lys)_2$, $Cu(His)_2$ or CuTyr complexes.

These data support earlier suggestions [10, 12, 16] that Cu is not bound to sulphur since no superoxide dismutase activity was observed using sulphur containing amino acid residues. Furthermore, at pH 8.0 [10] and more expressed at pH 11.8 [3, 16] superhyperfine splitting is observed in the EPR spectrum indicating possible coordination with several nitrogen atoms. From this phenomenon it was presumed that basic amino acids could perhaps be involved in the binding of Cu^{2+} although oxygen atoms derived from tyrosine residues cannot be excluded at the moment. Indeed $Cu(Lys)_2$ displayed the highest enzymic activity. Within the protein portion the chelated Cu^{2+} ought to be in an exposed position. It is very attractive to come to this conclusion since the second order rate constant of the enzymic catalysed disproportionation of $O_2^- \cdot$ [2] is near to the upper limit expected for a diffusion-controlled enzyme–substrate reaction and X-ray photoelectron studies revealed the easy access of Cu to the $Mg_{K\alpha}$-radiation [17].

The question remains open as to which degree the low molecular weight Cu^{2+}-chelates and the native enzyme react with identical reaction velocities. It is possible that the lower enzymic activities of the Cu–amino acids could be attributed to a diminished reaction velocity. This problem is currently under investigation.

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