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FUNCTIONAL ASPECTS OF THE SUPEROXIDE DISMUTATIVE ACTION OF Cu-PENICILLAMINE

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Summary

The superoxide dismutative action of Cu-penicillamine was examined by pulse radiolysis. The second order rate constant of the reaction with superoxide was $0.4 \pm 0.2 \cdot 10^9\ M^{-1} \cdot s^{-1}$, comparable to the action of Fe- and Mn-superoxide dismutases. No marked pH-dependence was seen. Neither ethylene diamine tetraacetic acid nor cyanide affected the catalytic action of Cu-penicillamine. The cyanide resistant reactivity as well as further X-ray photoelectron spectrometric measurements supported the suggestion of a Cu(I) stabilized sulphur radical being the active species involved in the catalysis of superoxide dismutation.

Introduction

Cuprein-bound copper has been shown to accelerate spontaneous superoxide dismutation by more than three orders of magnitude [1—5]. Due to this reactivity cuprein isolated from bovine erythrocytes by Mann and Keilin [6] was termed superoxide dismutase [1]. This functional phenomenon is not restricted to the protein-bound copper. The aquo-complex and the copper chelates of some amino acids [7—9] and salicylates [10—11] exert exactly the same catalytic activity. Moreover, $Cu(Tyr)_2$ [12] and the Cu-salicylates [10] have been successfully employed to examine the generation of superoxide ions in those metabolic sites to which the large cuprein molecule has no access. It was deduced that all chelated copper is capable of reacting with bound or free

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superoxide in essentially the same way. One important advantage of the superoxide dismutase active cuprein copper was its higher resistance to ethylene diamine tetraacetic acid treatment.

To improve possible applications of low molecular weight copper chelates provided with superoxide dismutase activity for analytical and/or therapeutical purposes, we were searching for a copper complex of a higher resistance to strong chelators. Preliminary studies [13—16] revealed the marked stability of Cu-penicillamine in the presence of ethylene diamine tetraacetic acid using indirect assays for superoxide dismutase activity. An interesting proposal was made in that the coordinated sulphur is the species undergoing the redox cycle rather than the liganded copper which remained in the $3d^{10}$ oxidation state.

In this context a detailed study was performed in which Cu-penicillamine and superoxide generated by pulse radiolysis were employed. The superoxide dismutase activity was examined at different pH values and in the presence of the strong chelator ethylene diamine tetraacetic acid. Special emphasis was placed on the redox state of the sulphur-coordinated copper using X-ray photoelectron spectrometry. Futhermore, the effect of cyanide, known to react with superoxide dismutase active Cu(II), on the Cu-penicillamine dependent catalysis was measured.

Materials and Methods

All chemicals employed were of analytical grade purity. D-penicillamine was from Serva, Heidelberg; $Cu(CH_3COO)_2 \times H_2O$, ethylene diamine tetraacetic acid (sodium salt), KCN, ethylenediamine, sodium formate, KOH and $HClO_4$ from Merck, Darmstadt; Sephadex G-10 from Pharmacia, Uppsala; Chelex-100 (sodium form) and Biogel P-6 from Biorad, Richmond. Triply distilled and pyrrolized water was used for pulse radiolysis experiments.

The red-violet copper-penicillamine complex was prepared as described in [13—15] and purified employing Chelex-100 and Sephadex G-10. Cu(ethylendiamine)$_2$(SCN)$_2$ was synthesized following the method given in Ref. 17. C,H,N-analyses were carried out employing a Perkin-Elmer 240 Elemental Analyzer unit. Cu was quantitated by atomic absorption spectrometry (Perkin-Elmer 400 S equipped with a HGA 76B Programmer). The values obtained were in accordance with the theoretical values (deviations lay within 3%). The molecular weight of Cu-penicillamine determined by gel chromatography on Biogel P-6 was 2200, the molar absorption coefficient ϵ_{535} was 1940 $M^{-1} \cdot cm^{-1}$. X-ray photoelectron spectra were run on a Varian V-IEE 15 high resolution electron spectrometer equipped with an on-line 620 L computer (8 K) as described in earlier studies [13—15].

Pulse radiolysis experiments were carried out using a Febetron unit. A xenon lamp, Osram XBO 450 W4, served as light source. In some of the experiments the optical detection system was composed of a Zeiss M4 QIII monochromator, an EMI 9659 QB photomultiplier and a Tectronix 7704 oscilloscope. Oscilloscope traces showing the decay of $\dot{O}_2^-$ at 250 nm were photographed with a Polaroid camera. In another set of experiments the oscilloscope and the camera were replaced by a transient recorder DATALAB 905. 40 ns pulses of high-energy electrons (1.81 MeV) were employed in oxygen saturated solutions

containing 10^{-2} M formate. The G-value of $^{\cdot}O_2^-$ was raised thus to 6.1. Superoxide concentrations were estimated employing thiocyanate dosimetry [18]. All rate constants represent the constants of second order reactions (k_2). In the cases of spontaneous superoxide dismutation, evaluation of the decay kinetics of superoxide radicals resulted in second order type reaction directly. When copper-penicillamine was present, the reactions showed pseudo first order type kinetics, from which, depending on the concentration of the complex, the second order rate constants were calculated using a Wang 2200 computer. For further experimental details see Refs. 8, 9 and 11.

The solutions employed were adjusted to the desired pH by addition of either KOH or $HClO_4$. Cu-penicillamine was added to the 1-ml solutions using Hamilton syringes directly before the start of the pulse radiolysis experiment. No buffers were used to avoid possible side reactions, however, pH control was maintained. Control experiments were carried out in the absence of formate to ensure that formate did not intervene with the reactions investigated.

Results

The redox state of copper

Originally, the employed $[Cu_{14}(\text{D-penicillamine})_{12}C]^{5-}$ complex was assigned to be a Cu(I)/Cu(II) mixed valence complex as deduced from X-ray difraction studies [19,20] Six out of the fourteen Cu ions are present in a square planar environment commonly found for Cu(II). The high absorption in the blue region allowed the proposal of some similarity with the type 1 copper chromophores. The phenomenon that S → Cu charge transfer transitions do occur in both the blue copper proteins and the Cu-penicillamine complex implicates that the actual electronic state of the copper should be considerably affected. As in earlier work [13—15] X-ray photoelectron spectrometric measurements of the Cu $2p_{3/2}$-level gave a binding energy value of 933.4 eV and no satellite was detectable. Thus it can be concluded that regardless of the molecular geometry essentially all copper in the Cu-penicillamine complex is present in the $3d^{10}$ electronic state, having the actual oxidation state +1 as defined by Jørgensen [21].

It may be argued that sulphur coordination to $3d^9$ copper may facilitate initial photoreduction of the metal during X-ray photoelectron spectrometry. Alternatively, a dramatic line broadening of the Cu $2p_{3/2}$ satellites might occur when $3d^9$ Cu(II) is bound to sulphur. Either theory might be favoured as we have always seen $3d^{10}$ Cu when this metal was attached to sulphur of low oxidation states. By way of contrast, the ease of polarization of the liganded sulphur was our explanation. The suggested polarization of the liganded sulphur is supported by the relatively higher sulphur 2p-binding energy near 163 eV (Fig. 1). Usually salt-like thiolate sulphur has a S 2p-value lower than 162 eV [23]. Furthermore, the EPR spectra of a frozen aqueous solution of Cu-penicillamine consists of a weak single broad band at $g \approx 2$ [20]. Cu- or ligand-hyperfine splitting are absent. Either measurement is consistent with the conclusion of a considerable electron delocalization and a high degree of covalent bonding in this copper-thiolate cluster.

Provided a second electronegative ligand is bound to the sulphur, $3d^9$ Cu

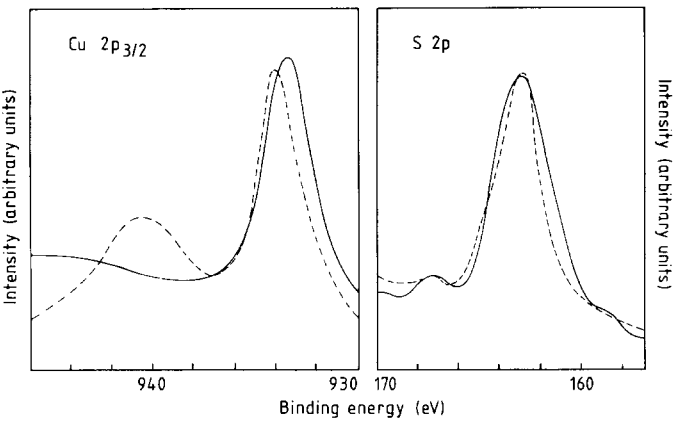

Fig. 1. X-ray photoelectron spectra of both the Cu $2p_{3/2}$ and the S 2p levels of Cu-D-penicillamine (———) and Cu(ethylenediamine)$_2$(SCN)$_2$ (- - - - -). Recording conditions: work function 6.0 eV; analyzer energy 100 eV; sweep width 20 eV; sweep time 20 s; number of channels 200; pressure range 1—2 μTorr; number of scans 10 in all cases. The samples were cooled during the measurements using liquid nitrogen.

should be stabilized. The usual ligand-$3d^9$ Cu interactions which are also known as shake up processes arising from delocalized binding electrons always produce more or less split satellites attributable to Cu(II) (see also Refs. 13—15).

An appropriate complex to shed some light on this problem appeared to be Cu(ethylenediamine)$_2$(SCN)$_2$ as from X-ray crystallographic data the sulphur coordination to the copper is known [17]. In this complex the copper retained its $3d^9$ oxidation state even upon prolonged X-ray photoelectron spectrometric measurements (Fig. 1). Thus, photoreduction of our earlier examined Cu-S-compounds including the Cu-penicillamine complex must be definitely excluded. The weakened Cu(II)-sulphur interaction in the Cu(ethylenediamine)$_2$(SCN)$_2$ complex is facilitated by the greater distance of the Cu-SCN bond (3.27 Å) resulting from the Jahn-Teller-distortion. The dislocation of the sulphur binding electrons in the other direction towards the cyanide moiety is seen by the distinct low N 1s binding energy of 398 ± 1 eV in the present and many other SCN^--salts [22]. Usually the N 1s-values are around 400 eV [13,14, 23].

The reaction with superoxide

Cu-penicillamine exhibited a marked superoxide dismutase activity in the cytochrome *c* reductase and the nitroblue tetrazolium assays [15] as well as during the oxidation of hydroxylamine and the ethylene production by isolated chloroplasts [16]. All these indirect assays do not allow the conclusion of a direct reaction of superoxide with this copper complex. To demonstrate its genuine superoxide dismutase activity $^{\cdot}O_2^-$ was generated pulse radiolytically. The decay of $^{\cdot}O_2^-$ was monitored at 250 nm. The second order rate constant of the spontaneous decay was $2.3 \pm 0.3 \cdot 10^6\ M^{-1} \cdot s^{-1}$. In the presence of variable concentrations of Cu-penicillamine markedly higher second order rate constants were determined. k_2 was highest ($6 \cdot 10^9\ M^{-1} \cdot s^{-1}$) using 10^{-8} M Cu-penicillamine and dropped to a constant value of $4.5 \pm 0.5 \cdot 10^8\ M^{-1} \cdot s^{-1}$ in the

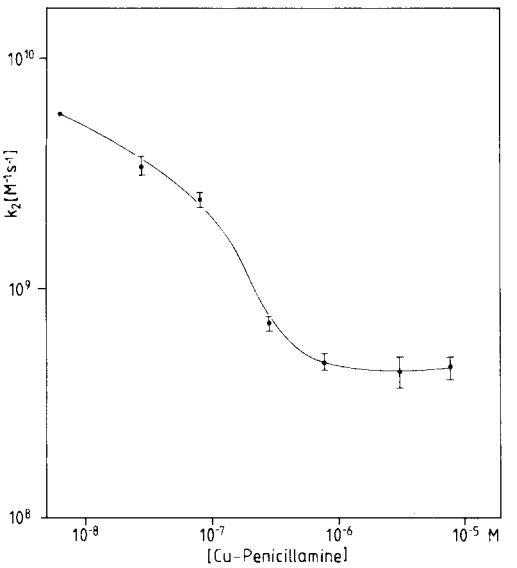

Fig. 2. The second order rate constant (k_2) of the reaction between Cu-penicillamine and $^{\cdot}O_2^-$ at different concentrations of the copper chelate. Oxygen saturated solutions in triply distilled and pyrrolized water containing 10^{-2} M formate were employed. The pH was adjusted to 7.0. A Febetron pulse radiolysis unit was used to generate 40 ns electron pulses of 1.81 MeV. The decay of superoxide was followed by observed the oscilloscope traces at 250 nm using a Tectronix 7704 oscilloscope equipped with a Polaroid camera. Alternatively, a transient recorder DATALAB 905 was used. The k_2-values were calculated employing a Wang 2200 computer. In the absence of the chelate k_2 was measured directly, while in the presence of Cu-penicillamine it was calculated from the pseudo first order rate constants.

presence of μmolar or higher concentrations (Fig. 2).

It should be pointed out that the Cu-penicillamine catalyzed superoxide dismutation proceeds with essentially the same velocity as observed with many known superoxide dismutases ($k_2 = 2.4 \cdot 10^9$ to $5.5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) [2—5,24, 25]. The significant rise of k_2 at 10^{-7}—10^{-8} M concentrations of Cu-penicillamine could be attributed to the dissociation of extraneous Cu(II) from the outer sphere of the cluster. This disturbing reactivity should be countered by the addition of ethylene diamine tetraacetic acid. This strong chelator is usually added to the different native superoxide dismutases to avoid uncontrolled side reactions of unspecifically bound transition metals (Fig. 3).

The chelator alone affected the $^{\cdot}O_2^-$ decay. From 10^{-7} M on to higher concentrations of ethylene diamine tetraacetic acid suppressed the spontaneous $^{\cdot}O_2^-$-dismutation. However, in the presence of 10^{-8}—10^{-6} M Cu-penicillamine the catalysis of superoxide dismutation remained constant at $2 \pm 2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$. The apparent discrepancy between the data of Figs. 2 and 3 is due to an overlapping of the reaction of superoxide with the complex and the direct reaction of ethylene diamine tetraacetate with $^{\cdot}O_2^-$. This favours the assumption that dissociated spurious Cu(II) from the complex has been trapped by the chelator. The diminished rate constant caused by 10^{-6} M or higher ethylene diamine tetraacetic acid concentrations must be assigned to the reactivity of the free chelator and is not connected to the chelation of metal impurities.

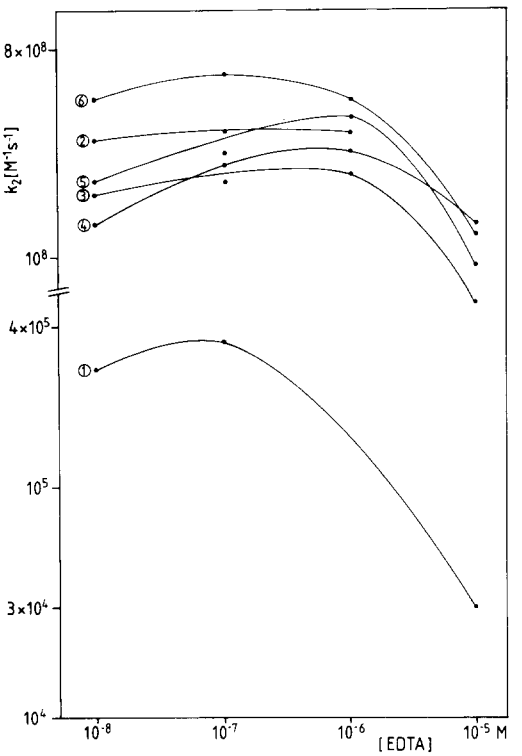

Fig. 3. Effect of different concentrations of ethylene diamine tetraacetic acid (EDTA) on the rate constant of the reaction between Cu-penicillamine and $^{\cdot}O_2^-$. 1, EDTA alone; 2—6, +Cu-penicillamine in the concentrations: 2, $9.1 \cdot 10^{-8}$ M; 3, $4.8 \cdot 10^{-7}$ M; 4, $9.1 \cdot 10^{-7}$ M; 5, $4.8 \cdot 10^{-6}$ M and 6, $9.1 \cdot 10^{-6}$ M. Experimental details are the same as for Fig. 2. Higher inhibitor concentrations were not examined due to both the uncontrolled reactivity of the ligand with $^{\cdot}O_2^-$ and the increase in viscosity of the solutions leading to erroneous rate constants.

The pH-dependence of the Cu-penicillamine catalyzed superoxide dismutation reveals no dramatic changes of k_2. Unlike the strong pH-dependent spontaneous reactions from $3 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ (pH 3) to $5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ (pH 9) the same pH-variations of Cu-penicillamine solutions resulted in rate constants still being in the range of the usual enzymic catalyzed superoxide dismutation. However, some acceleration was observed at the pK of the $^{\cdot}O_2^-/HO_2^{\cdot}$ system (Fig. 4). Added ethylene diamine tetraacetic acid had no effect on the k_2-values of the Cu-penicillamine catalyzed reaction at different pH (Fig. 5). As in Fig. 2 the spontaneous $^{\cdot}O_2^-$-disproportionation was diminished starting from pH 5 onwards.

The increased rate constants at lower pH-values might be due to the dissociation of the Cu-penicillamine cluster in some active small molecular weight species. It is certainly not spurious copper as this would have been trapped by the added ethylene diamine tetraacetate.

X-ray photoelectron spectrometric studies suggested that throughout the reaction of Cu-penicillamine with $^{\cdot}O_2^-$ the copper remained in the $3d^{10}$ (Cu(I))

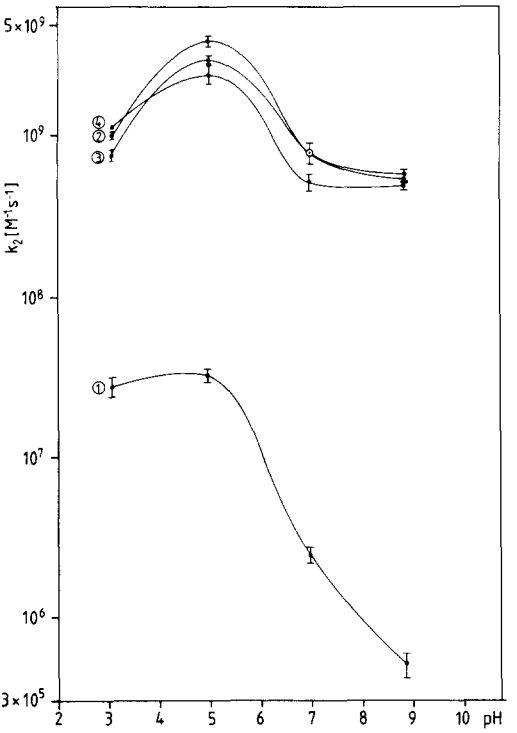

Fig. 4. pH-dependend of the rate constant of the reaction between $^{\cdot}O_2^-$ and Cu-penicillamine. Cu-penicillamine concentrations: 1, 0; 2, $4.8 \cdot 10^{-7}$ M; 3, $9.1 \cdot 10^{-7}$ M; 4, $4.8 \cdot 10^{-6}$ M. The experimental procedure is described in the legend to Figs. 2 and 3.

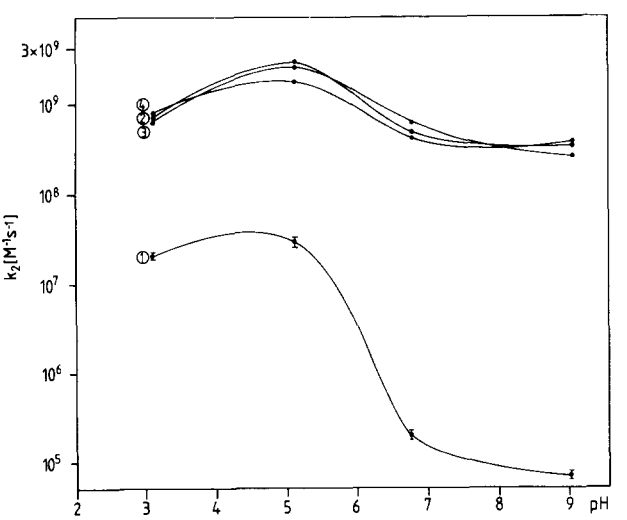

Fig. 5. pH-dependence of the rate constant of the reaction between superoxide and Cu-penicillamine in the presence of 10^{-6} M EDTA. Cu-penicillamine was present in the concentrations: 1, 0; 2, $4.8 \cdot 10^{-7}$ M; 3, $9.1 \cdot 10^{-7}$ M; 4, $4.8 \cdot 10^{-6}$ M. For experimental details see legend to Figs. 2 and 3.

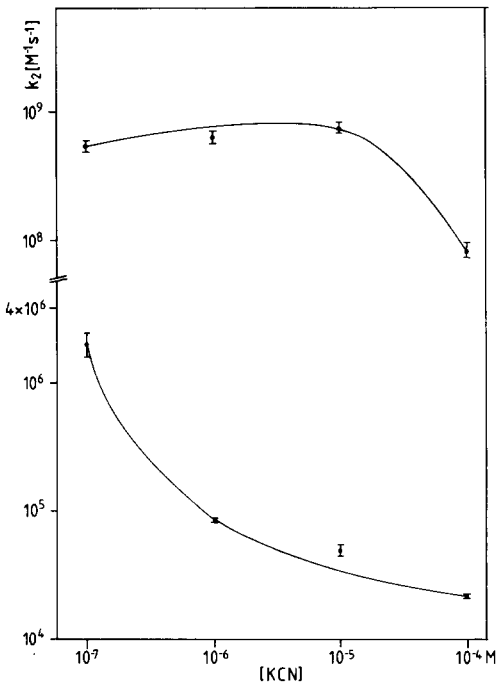

Fig. 6. Effect of added cyanide on the rate constant of the reaction of $^{\cdot}O_2^-$ with Cu-penicillamine. Lower curve: spontaneous decay of superoxide; upper curve: decay of $^{\cdot}O_2^-$ in the presence of $9.1 \cdot 10^{-6}$ M Cu-penicillamine. Experimental details are given in the legend to Figs. 2 and 3.

stat. Thus, the actual electron transfer ought to have taken place according to:

$$Cu(II)^-SR \rightleftharpoons Cu(I)^{\cdot}SR$$

$$Cu(I)^{\cdot}SR + {}^{\cdot}O_2^- \rightarrow Cu(I)^-SR + O_2$$

$$Cu(I)^-SR + {}^{\cdot}O_2^- \rightarrow Cu(I)^{\cdot}SR + O_2^{2-}$$

Provided this reaction mode is favoured, added cyanide should not influence the catalysis of superoxide dismutation. Cyanide would only bind to the copper, if at all, leaving the redox active sulphur radical unaffected. Encouraging support for this suggestion was already obtained during the crocin destruction by enzymically generated superoxide [16].

In this study, crocin, the digentobiose of the carotenoid acid crocetin was used as a model compound for studies of carotene bleaching by oxygen radicals produced by the xanthine-xanthine oxidase system. Excessive cyanide did not inhibit the action of Cu-penicillamine in this assay. Using KO_2, a pure inorganic source of superoxide, for the reduction of nitroblue tetrazolium [26] the superoxide dismutase activity of Cu-penicillamine proved to be constant in the presence of a 20-fold excess of cyanide.

The same resistance of the Cu-penicillamine complex against cyanide was seen in the electron absorption spectrum. No detectable changes of the absorption bands could be measured. The spontaneous decay of $^{\cdot}O_2^-$ was considerably affected in the presence of free cyanide (Fig. 6). It was ascertained that the

diminished second order rate constant is not attributable to pH changes. Special care was taken to adjust the pH to 7 before $^{\cdot}O_2^-$ was generated by the 1.8 MeV electron irradiation. As long as no free cyanide was available (i.e. 10^{-7}—10^{-5} M CN^-) the Cu-penicillamine catalysis remained constant. Upon the addition of 10^{-4} M cyanide a reduced rate constant (k_2) was calculated. An overlapping of the reaction of free cyanide with $^{\cdot}O_2^-$ can be considered to be the cause of this phenomenon. Nevertheless, as in the indirect assays for superoxide dismutase activity, excessive cyanide did not affect the Cu-penicillamine catalysis. This is contrasted by the strong cyanide dependent enzymic activity of native 2 Cu, 2 Zn-superoxide dismutase.

Discussion

Many low molecular weight copper compounds were shown to exhibit a marked superoxide dismutase activity in earlier studies [7—11]. The discovery that Cu-penicillamine mimics perfectly the activity of native cuprein in catalyzing the spontaneous dismutation of superoxide radicals is of far reaching significance in two aspects. First, its reaction with $^{\cdot}O_2^-$ is as efficient as with many a native superoxide dismutase, which could be judged by the high second order rate constant. Second, this catalytic activity is maintained in the presence of high concentrations of the strong chelator ethylene diamine tetraacetic acid. This finding implies that Cu-penicillamine will survive in biological fluids and exert its superoxide dismutating action at a cellular level, where strong biogenic chelators are present.

In contrast to the known low molecular weight superoxide dismutating complexes [7—11], the mode of action of Cu-penicillamine is of a different type. Here, the actual redox-system reacting with superoxide is suggested to be $RS^{\cdot}/RS^-$. A chalcogen, sulphur, replaced the metal usually present in native superoxide dismutases. For the polarization of sulphur and/or to stabilize the respective redox states Cu(I) appears to be a convenient metal. Polarized chalcogens are more widely distributed redox systems in cellular biochemistry than originally thought. For example, unlike sulphur, selenium can readily be polarized serving thereby as a perfect electron donor. Thus, it is not surprising to find selenium involved in peroxide metabolism. In glutathione peroxidase [27] the protein bound selenium needs no metal for stabilizing its redox active form. From Mössbauer spectroscopic data of iron-sulphur proteins it was seen that during electron transfer the cysteine sulphur and not the iron participates in the electron transport (Gersonde, K., personal communication).

The antiinflammatory activity exhibited by Cu-penicillamine is a phenomenon of major interest. The therapeutic action was seen at an initial screening dose at which penicillamine alone was inactive [28]. The stability of the cluster offers the advantage that no dissociation would occur so that the antiinflammatory activity would not be altered during clinical application.

One should be, however, be cautious, penicillamine is known to bind strongly to copper in vivo as could be judged by its high cupriuric activity [29]. It would, thus, bind to biogenic copper present in many Cu-proteins and enzymes, such as the amine oxidases and cytochrome *c* oxidase, causing thereby major physiological damage. The Cu-penicillamine-cluster, remaining

stable in the cell, might also capture $^{\cdot}O_2^-$-radicals which are produced during enzymic oxidation and oxygenation reactions, and so inhibit many a metabolic pathway where the $^{\cdot}O_2^-$-intermediate is essential.

On the other hand, due to its much higher stability, Cu-penicillamine offers many advantages over the known low molecular weight Cu-chelates provided with a superoxide dismutase activity as a probe to prove the participation of superoxide in enzymic reactions. $(Cu(Tyr)_2$ and the Cu-salicylates were successfully employed to show the involvement of $^{\cdot}O_2^-$ during cytochrome *P*-450 dependent reactions [12] and during the diamine oxidase catalysis [30]. The inhibitory action of Cu-D-penicillamine on prostaglandin biosynthesis was even much higher than that of the other superoxide dismutating copper complexes (unpublished data). This shows that, as a consequence of its stability, the Cu-penicillamine cluster is a valuable tool to detect superoxide where other indicators fail to do so due to their large molecular dimensions (i.e native superoxide dismutases) or their low stability in the presence of strong chelators.

Acknowledgements

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