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Decomposition and Superoxide Dismutase Activity of the Copper Complex of D-Penicillamine ($Cu(II)_6Cu(I)_8(D\text{-Penicillamine})_{12}Cl)^{5-}$

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Abstract

The possible time- and/or light-dependent decomposition of the purple Cu(I), Cu(II)-complex of D-penicillamine $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was examined. Superoxide dismutase activity of the freshly prepared complex was assayed using the nitroblue tetrazolium assay. The formazan colour formation was inhibited by 50% in the presence of approximately 500 μM copper. Ageing of the copper complex, especially in the light, resulted in a marked increase of EDTA-sensitive activity. Upon gel chromatography of the aged samples the original low inhibitory activity was restored. All EDTA-sensitive inhibitory activity was found in a clearly separated low M_r copper-containing fraction. Aerobic irradiation with a tungsten lamp at 30 °C accelerated the decomposition of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$. $\epsilon_{Cu518} = 1800\ M^{-1}\ cm^{-1}$ dropped to $\epsilon_{Cu640} = 60\ M^{-1}\ cm^{-1}$. The photochemical conversion of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was complete within 48 h. Due to the identical electronic absorption profile of both, the decomposition product and Cu(II) D-penicillamine disulphide the latter complex was assigned to be the unknown low M_r copper-compound. Circular dichroism and electron paramagnetic resonance measurements support this conclusion.

Introduction

Attributable to its strong metal-chelating activity, D-penicillamine is used for treatment of Wilson's disease to remove copper excessively deposited in many tissues [1, 2]. Furthermore, it is applied in liver disorders accompanied by hepatic accumulation of copper, for instance cholestasis, hepatitis, primary biliary cirrhosis, and neonatal jaundice [3, 4]. Renal excretion of copper is promoted by D-penicillamine [1]. It is also widely employed as an antirheumatic agent [5, 6]. Possible antiinflammatory, antiarthritic, and antiulcer activity of different copper complexes of D-penicillamine has been described [7, 8].

Antirheumatic activity has been assigned to mixed disulphide formation [9] and/or the possible superoxide dismutase activity of copper complexes formed *in vivo* [10]. The latter reactivity has been studied using the crystalline purple mixed-valence complex $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ [10–12]. This complex exerted superoxide dismutase activity in the nitroblue tetrazolium assay [10]. The first order rate constant for the catalysis of superoxide dismutation (determined by pulse radiolysis) was $0.4 \pm 0.2 \times 10^9\ M^{-1}\ s^{-1}$ and was comparable to the action of Fe- and Mn-superoxide dismutases [12]. No such reactivity was seen in the cytochrome *c* reductase assay [13]. The discrepancy in the respective superoxide dismutase activities might be because the copper penicillamine complexes used were not identical. Thus, it was of interest to examine in more detail the chemical stability of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$. The photochemical reactivity deserved special attention. Time-dependent irradiation experiments were carried out. Possible copper complexes generated during irradiation from $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ were monitored by gel chromatography, electron absorption spectrometry, circular dichroism and electron paramagnetic resonance (EPR) measurements. The superoxide dismutase activity was followed employing the nitroblue tetrazolium assay.

Experimental

Materials

D-Penicillamine, D-penicillamine disulphide, and bovine serum albumin were purchased from Sigma, München, and used without further purification. Xanthine oxidase from Serva, Heidelberg, was freed from EDTA and salicylate by passage through Sephadex G-50. All other chemicals were of reagent grade quality.

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Preparations

$(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was prepared from $CuCl_2$ and D-penicillamine as described by Birker and Freeman [14]. Prior to use the dissolved complex was additionally purified by Sephadex G-25 chromatography on a 1.4 × 42 cm column. ϵ_{Cu518} was determined to be 1800 M^{-1} cm^{-1}. Equimolar concentrations of copper sulphate and D-penicillamine disulphide were combined to yield an aqueous solution of Cu(II) penicillamine disulphide.

Gel Filtration

Gel filtration was performed on Sephadex G-25 (column size 1.4 × 42 cm). Elution was carried out with water or 20 mM HEPES, pH 7.4, containing 150 mM sodium chloride. 3 ml fractions were collected. The progress of the separation was monitored at A_{280} and by atomic absorption spectrometry.

Irradiation Experiments

Irradiation of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was performed with a Schott KL 105 B cold light source equipped with a 150 W tungsten lamp Osram Xenophot HLX and an IR-reflecting filter Schott KG 37. The sample was positioned 14 cm apart from the light source. Temperature was maintained at 30 °C.

Nitroblue Tetrazolium Reduction Assay

Superoxide dismutase activity was assayed by inhibiting the reduction of nitroblue tetrazolium, using xanthine/xanthine oxidase as a superoxide source. The test system contained in 0.5 ml: 20 mM HEPES, pH 7.4; 150 mM sodium chloride; 0.18 μM xanthine oxidase; 50 μM xanthine; 0.8 μM catalase, 0.62 mM nitroblue tetrazolium chloride; 0.2% (w/v) gelatine. Formazan formation was monitored at 540 nm and 23 °C in 4 × 10 mm cells using a Beckman spectrophotometer Model 25.

Determination of Half-Inhibition of Nitroblue Tetrazolium Reduction

Inhibition of nitroblue tetrazolium reduction was determined at three different copper concentrations each. The copper concentration required for 50% inhibition (corresponding to 1 unit of superoxide dismutase activity) was retrieved by linear regression analysis, taking the logarithm of the copper concentration.

Spectrometry

Electron absorption spectra were recorded on a Beckman spectrophotometer Model 25. Circular dichroism measurements were performed on a Jasco J 20 A spectropolarimeter. Electron paramagnetic resonance was measured at 100 K on a Varian E 109 EPR spectrometer. Copper was determined using a Perkin-Elmer 400 atom absorption spectrometer furnished with a HG 76 B graphite furnace.

Results and Discussion

Before superoxide dismutase-like activity was measured, freshly prepared $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was additionally gel-filtrated on Sephadex G-25. The purple copper complex migrated in one homogeneous peak. Superoxide dismutase measurements were carried out immediately. Unlike the micromolar concentrations of copper used in earlier work [10], more than 500 μM became necessary to inhibit by 50% the xanthine/xanthine oxidase-mediated reduction of nitroblue tetrazolium. The same result was obtained when the $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was gel-filtrated in water or 20 mM HEPES buffer, pH 7.4 containing 150 mM NaCl. Intriguingly, within one day at room temperature the reactivity of the copper complex rose more than one order of magnitude ($C_{Cu,50\%}$ 15 μM) (Fig. 1). However, this rise was markedly diminished in the dark. Thus, light was found to be a major factor to induce the inhibitory activity. Unlike in the absence of oxygen the activity appeared to increase slightly faster under aerobic conditions.

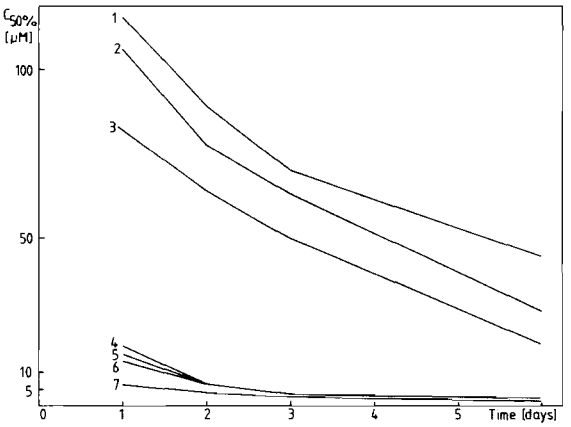


Fig. 1. Time-dependence of the inhibitory activity of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ in the nitroblue tetrazolium reduction test. $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ gel-filtrated over Sephadex G-25 was divided into seven portions. Samples **1–3** were stored in the dark, **4–6** were kept in the presence of daylight, **7** was irradiated with a 150 W tungsten lamp Osram Xenophot HLX. The temperature was 23 °C. Samples **1** and **4** were purged with nitrogen for 30 min and stored under nitrogen; samples **3** and **6** were kept under oxygen; samples **2, 5** and **7** were stored in the presence of air. Aliquots were taken after the indicated times and copper concentrations required to yield 50% inhibition ($C_{50\%}$) of nitroblue tetrazolium reduction were determined. The standard errors of copper determination and assay of the inhibition of nitroblue tetrazolium reduction were less than 5%. $C_{Cu,50\%}$ immediately after gel filtration (0 days) was 570 μM copper.

After the addition of 200 μM EDTA and/or 560 μM bovine serum albumin the inhibitory activity of the copper complex was abolished. We emphasize that the $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$, estimated by its absorbance at 518 nm, survives these chelators in the dark without any signs of decomposition for weeks. Thus, a small fraction of the copper complex not detectable by absorption spectrometry could be the active component which, however, is not stable in the presence of strong chelators.

A preparative approach was carried out to concentrate and separate this component. Owing to the observed light-induced increase of activity $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was irradiated using a 150 W cold light source. Gel filtration of an irradiated sample revealed a significant decrease of the major purple elution band with the concomitant appearance of a clearly separable low M_r component. Essentially all $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ had decomposed within 50 h; only the low M_r compound remained (Fig. 2). The strong inhibitory

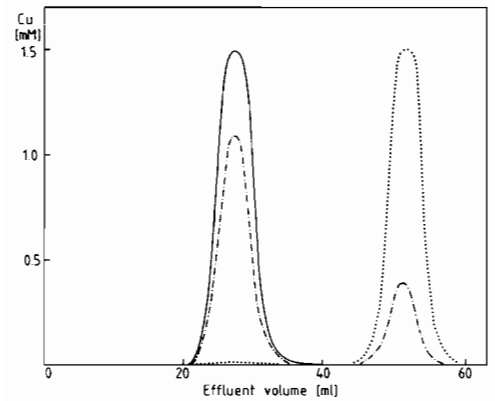
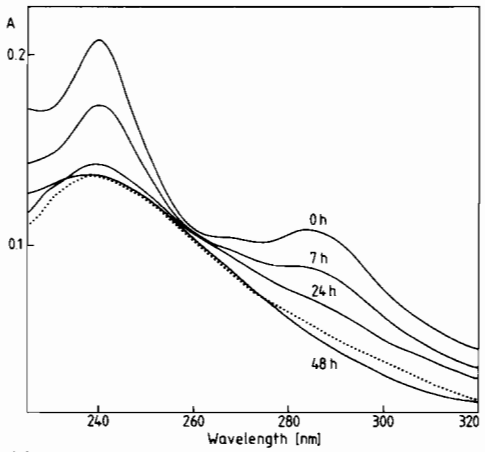


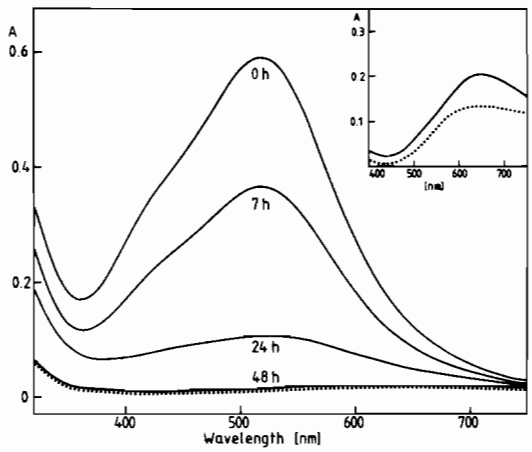
Fig. 2. Gel chromatography of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ previously irradiated at different periods of time. Freshly prepared $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was irradiated with a 150 W tungsten lamp and subjected to gel filtration on Sephadex G-25 (1.4 × 42 cm). Control (———); irradiation for 16 h (–·–·–); 48 h (·····).

activity was exclusively assigned to this low M_r fraction while the mixed valence copper complex found in the higher M_r fraction had the substantially diminished activity of freshly prepared $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$, where approximately 500 μM of copper were required to inhibit the nitroblue tetrazolium reduction.

The same irradiation experiments were spectrometrically controlled. For example, the course of the decomposition was recorded by electron absorption spectrometry (Figs. 3 a, b). The major absorption band at 518 nm was progressively diminished and a redshift of the maximum towards 640 nm was observed. After 48 h the strong absorbance of ϵ_{Cu518}



(a)

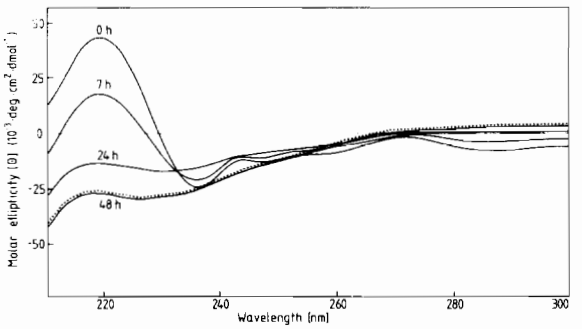


(b)

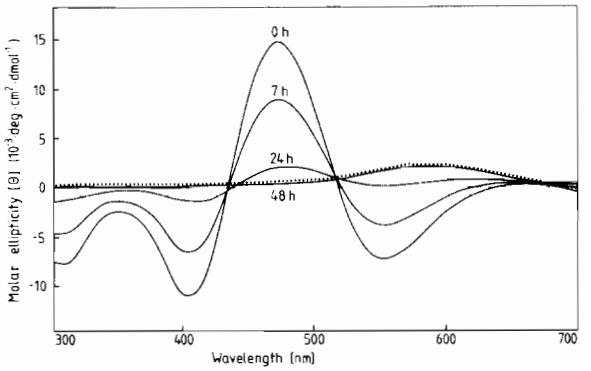
Fig. 3. Ultraviolet (a) and visible (b) electronic absorption of irradiated $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ and Cu(II) D-penicillamine disulphide. Freshly prepared $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ in aqueous solution (3.3 mM copper) was treated as in the legend to Fig. 2. Spectra were recorded at the times given at the respective curves. The dotted line (·····) represents the spectrum of Cu(II) D-penicillamine disulphide. All samples contained 0.33 mM copper; 18 mM HEPES, pH 7.4; 135 mM sodium chloride. In the inset a tenfold higher concentration of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ irradiated for 48 h (———), and Cu(II) D-penicillamine disulphide (·····), respectively, was used.

= 1800 M^{-1} cm^{-1} levelled off and a nonblue-type absorption of ϵ_{Cu640} = 60 M^{-1} cm^{-1} was detectable. In the ultraviolet region the absorption band at 285 nm disappeared while a second band near 240 nm was reduced to half of the original magnitude.

It was very attractive to conclude that due to photochemically induced intramolecular redox reactions the penicillamine moiety might be converted into penicillamine disulphide. Therefore, the absorption profile of a separately prepared copper(II)



(a)



(b)

Fig. 4. Circular dichroism of irradiated $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ (———), and Cu(II) D-penicillamine disulphide (.....) in the ultraviolet (a) and visible (b) region. $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was treated as in legend to Fig. 2. The irradiation time is given at the respective curves.

D-penicillamine disulphide complex was recorded. An intriguing similarity with the copper complex obtained photochemically was noticed.

The results of circular dichroism measurements of the photochemical reaction were even more distinct (Fig. 4a, b). The negative Cotton extrema at 285, 310, 405 and 555 nm and the two positive bands at 220 and 470 nm disappeared. There is some indication of possible transient products as isosbestic points at 233 and 433 nm observed up to 24 h of irradiation are not intersected by the final product. At the end of the reaction, the chiroptical properties of Cu(II) D-penicillamine disulphide and the photochemically prepared copper complex were identical.

Of special interest was the region between 500 and 680 nm. The negative Cotton band of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ at 550 nm has been attributed to a S → Cu(II) charge transfer transition [15]. During the photochemical excitation an electron might be transferred from the thiolate sulphur to copper(II) accompanied by a cleavage of the Cu(II)–S bonding leading to the breakdown of the chromophore. The resulting thiyl radical could induce the oxidation of another sulphide group in its neighbourhood. Copper(I), the reduction product, might be reoxidized to copper(II) by oxygen and/or by disproportionation into copper(II) and elemental copper.

In fact, the negative Cotton band at 555 nm is reduced and replaced by a positive Cotton band somewhat red-shifted to 590 nm. The product of the photochemical disintegration of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ and Cu(II) D-penicillamine disulphide exhibited identical spectra.

Electron paramagnetic measurements supported the former conclusions of a photochemically formed copper(II) compound coordinated in a possible Cu(II) D-penicillamine disulphide complex (Fig. 5). The broad signal of $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ developed progressively into signals typical of common copper(II) compounds. The EPR parameters of Cu(II) D-penicillamine disulphide were essentially identical to those of the photochemically generated copper compound.

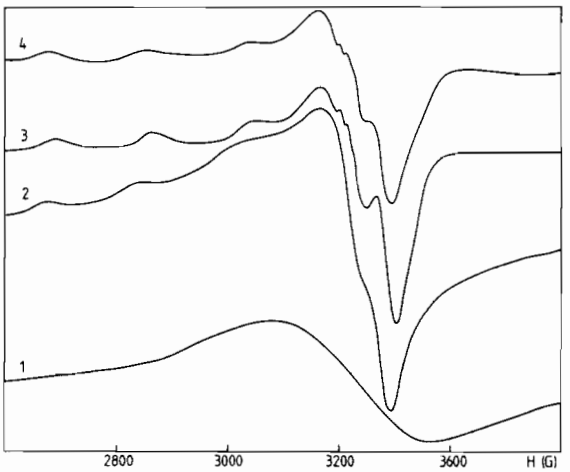


Fig. 5. Electron paramagnetic resonance of irradiated $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ and Cu(II) D-penicillamine disulphide. $(Cu(II)_6Cu(I)_8(D\text{-penicillamine})_{12}Cl)^{5-}$ was treated as in the legend to Fig. 2. Curves 1–3 represent spectra of samples irradiated for (1) 0 h, (2) 2 h, and (3) 48 h, respectively. Curve 4 represents the spectrum of Cu(II) D-penicillamine disulphide. Copper concentrations were 0.33 mM. The spectra were recorded at 100 K in frozen aqueous solution. Microwave power 20 mW, microwave frequency 9.24 GHz, modulation amplitude 10 G, modulation frequency 100 kHz. Receiver gain was: (1) 12500, (2) 10000, (3) 3200, and (4) 2000.

Conclusion

The apparent discrepancy of the reports on the superoxide dismutase-mimetic activity of $(Cu(II)_6$-

$Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ [10–13] must be seen in the light of the present results. The freshly prepared copper complex is unable to catalyse the spontaneous superoxide dismutation to a reasonable degree. Ageing of the dissolved complex, preferably under daylight, results in the formation of Cu(II) D-penicillamine disulphide. Upon irradiation with a tungsten light source this conversion is substantially accelerated and is completed within 48 hours. This amino acid-type copper(II) complex is highly active in the nitroblue tetrazolium assay. The superoxide dismutase-like activity of $(Cu(II)_6Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ of earlier work [10–12] may be attributed to traces of Cu(II) D-penicillamine disulphide simultaneously present in the assay solutions and indistinguishable by elemental chemical analysis. At the same time the strong electronic absorption of the $(Cu(II)_6Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ overlapped the extraordinarily low ϵ_{Cu640} of Cu(II) D-penicillamine disulphide near 60 M^{-1} cm^{-1}.

In biological systems the photochemical generation of Cu(II) D-penicillamine disulphide seems rather unlikely, except in plant or microbial systems. However, uncontrolled redox reactions attacking the $(Cu(II)_6Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ in cellular systems should be taken into consideration. In this regard 'photobiochemistry in the dark' might be brought forward [16]. Excited electronic states are not only attained by absorption of electromagnetic radiation, but might also be induced by chemical reactions. Owing to a detectable chemiluminescence 'chemienergized' species are assumed in phagocytosis by activated polymorphonuclear leukocytes, as well as in lipid peroxidation. Energy transfer from these excited species to $(Cu(II)_6Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ might be able to induce its decomposition.

In the early stage of penicillamine application $(Cu(II)_6Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ generated *in vivo* is capable to transport copper in a biochemically inactive form. This is the desired compound for removal of excessive copper concentrations from the tissue. However, with increasing time and under certain metabolic conditions this copper complex is expected to decompose and Cu(II) will again become biochemically active. This might be particularly true for inflammatory sites where phagocytes are known to accumulate.

The rise of serum copper in inflammatory diseases is commonly regarded as being beneficial to overcome inflammation [17]. Thus, the antirheumatic activity of $(Cu(II)_6Cu(I)_8$(D-penicillamine)$_{12}Cl)^{5-}$ might be seen in the initial strong chelating activity with the gradual possibly site-specific accumulation of reactive Cu(II) at a later stage. Unfortunately, the exact role of copper in inflammation is still not fully understood.

Acknowledgements

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