REACTIONS OF D-PENICILLAMINE WITH COPPER IN WILSON'S DISEASE

Heinz Rupp and Ulrich Weser*

Physiologisch-chemisches Institut der Universität
D-7400 Tübingen, Hoppe-Seyler-Strasse 1,Germany

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SUMMARY: X-ray photoelectron spectroscopy proved very appropriate to determine the genuine oxidation state of copper in copper(D-penicillamine) chelates. The present data corroborate the 'reductive chelation' mechanism proposed by Peisach and Blumberg (Mol.Pharmacol.5,200-209,1969) for the mobilization of Cu(II) with D-penicillamine (RSH). Moreover, it was concluded that the resulting complex $[RSCu(I)]_n$ is a suitable form for urinary excretion of copper.

The copper metabolism in Wilson's disease and in newborn infants is strikingly similar. Both have large quantities of copper in the liver which is contrasted by an unusually low ceruloplasmin level in the blood (1). In healthy humans these characteristics convert to normal within the first months after birth, whereas in homozygous carriers of the gene causing Wilson's disease this metabolic state persists leading to deposition of copper in abnormal amounts in the brain, cornea and kidneys (2). In the neonatal period a high portion of the physiologically increased copper is bound to neonatal hepatic mitochondrocuprein, a protein with a high copper and sulphur content(3). In previous studies (4, 5) we were able to elucidate the proposed relationship between mitochondrocuprein and metallothionein (6-8). From the liver of cases of Wilson's disease a copper protein with similar properties could be isolated by Porter (9). Morell et al. characterized the hepatic copper binding protein L-6-D (10-12) which is very similar to Cu-thionein (4). A common feature of these proteins is the tightly bound copper, probably Cu(I) coordinated with thiolate sulphur(12). In the course of the untreated disease excessive copper ions are also bound to various proteins which are normally free of copper.

The high cupriuric activity of D-penicillamine (ß,ß-dimethylcysteine)

*To whom all correspondence should be sent.

made this chelating agent to the drug of choice in the therapy of Wilson's disease (13). At present the mode of chelation with copper is not fully understood. Thus, it was very tempting to perform X-ray photoelectron spectroscopic measurements to determine the genuine oxidation states of copper in these complexes. Furthermore, D-penicillamine has also been suggested (14) for use in the treatment of rheumatoid arthritis, schizophrenia and cystinuria; a critical study of its possible reactions with copper appeared worthwhile.

MATERIALS AND METHODS

All chemicals employed were of reagent grade or better. D-penicillamine was from Serva, Heidelberg; $Cu(CH_3COO)_2 \cdot H_2O$ from Merck, Darmstadt; Sephadex G-10, G-25 from Pharmacia, Uppsala; Chelex-100 from Biorad, Richmond. The red-violet copper(D-penicillamine) complex was prepared by adding 0.666 mMol D-penicillamine under argon to an aqueous solution containing 0.50 mMol $Cu(CH_3COO)_2 \cdot H_2O$ and 1.50 mMol NaCl (reaction mixture, 1:0.75 ratio of D-penicillamine and Cu(II) (16)). The addition under argon of 0.166 mMol D-penicillamine to aqueous 0.50mMol $Cu(CH_3COO)_2$ and 1.50 mMol NaCl results in a brown-green colour (reaction mixture, 1:3 ratio of D-penicillamine and Cu(II)). Elemental analyses were obtained from samples treated with Chelex-100 (sodium form), filtered and desalted on Sephadex G-10. This fractionation procedure was repeated prior to lyophilization. Found: C,23.29; H, 4.30; O, 20.37; N, 6.60; S, 12.92; Cu, 29.33; Na, 1.19; Cl, 1.77%. The molecular weight of 2 200 was determined using the gel filtration technique (17). X-ray (Mg $K_{\alpha 1,2}$) photoelectron spectra of lyophilized samples were recorded at -100°C on a Varian V-IEE 15 spectrometer equipped with an 620 L on line computer (17,18). All spectra were standardized employing the C 1s line at 284.0 eV originating from the aliphatic hydrocarbons of the Cellotape (3M Company). The standard deviation of the energy values was 0.1 eV.

RESULTS

In the organism D-penicillamine may undergo different reactions.

$$2\ RS^- + A_{ox} \rightleftharpoons RSSR + A_{red} \quad (a)$$

$$RS^- + R'SSR' \rightleftharpoons RSSR' + R'S^- \quad (b)$$

In reaction (a) the drug is oxidized to D-penicillamine disulphide by electron acceptors A_{ox}. According to reaction (b) it may form mixed disulphides with cysteine. Penicillamine cysteine disulphide represents the major form of urinary excretion (19). Cysteine desulphhydrase does not attack the thiol group of D-penicillamine (20). Thus, we have to consider at least three reactive species for chelation of copper ions. Further progress in the characterization of the different chelates de-

pends on the possibility to assign the genuine oxidation state of copper. Direct informations in this respect cannot be derived from titrimetric or electrochemical methods. In our previous studies X-ray photoelectron spectroscopy developed into a powerful tool for the analysis of Cu(I), Cu(II) and mixed valence Cu(I)-Cu(II) compounds (17,18). In the X-ray photoelectron spectra of Cu(II) compounds satellite peaks are well resolved on the higher binding energy side of the Cu $2p_{1/2}$, Cu $2p_{3/2}$ main signals. The shape of the satellites depends on the nature of the ligand atoms. In Cu(I) compounds these characteristic satellites are absent(21). Therefore, X-ray photoelectron spectroscopy is a useful way for studying oxidation and reduction processes occurring during copper chelation by D-penicillamine. Well characterized are the copper chelates of D-penicillamine, when both metal ion and ligand are either in the reduced or oxidized state. D-penicillamine disulphide reacts with Cu(II) as in reaction (c).

$$RSSR + Cu(II) \rightleftharpoons RSSRCu(II) \qquad (c)$$

In blood a mixed ligand complex of Cu(II) with D-penicillamine disulphide and histidine may occur (22). Since oral administration of D-penicillamine disulphide does not promote excretion of copper(23), we must assume that D-penicillamine is the most active species in the mobilization of copper. Cu(I) forms with D-penicillamine a polymeric complex of high stability according reaction (d) (24).

$$RS^- + Cu(I) \rightleftharpoons \frac{1}{n}\left[RS^-Cu(I)\right]_n \qquad (d)$$

X-ray photoelectron spectra of the corresponding copper chelates of cysteine and cystine confirmed the presumed oxidation states of copper (17). Of great importance was the reaction of D-penicillamine with Cu(II). The addition of D-penicillamine to Cu(II) in the ratio 1:0.75 results in a deep-violet complex of molecular weight 2 200(17). Regarding the molecular structure of the copper chelate an unequivocal decision could not be reached in the past. The X-ray photoelectron spectrum of the lyophilized violet reaction mixture (Fig.1, ①) shows the presence of a large portion of Cu(I), although signals attributable to Cu(II) (shoulder at 936.0 eV, increased line width and satellite at 944.2 eV) are still detectable.

Thus, we can conclude that the formation of the complex $\frac{1}{n}\left[RSCu(I)\right]_n$ is favoured (reaction (e)).

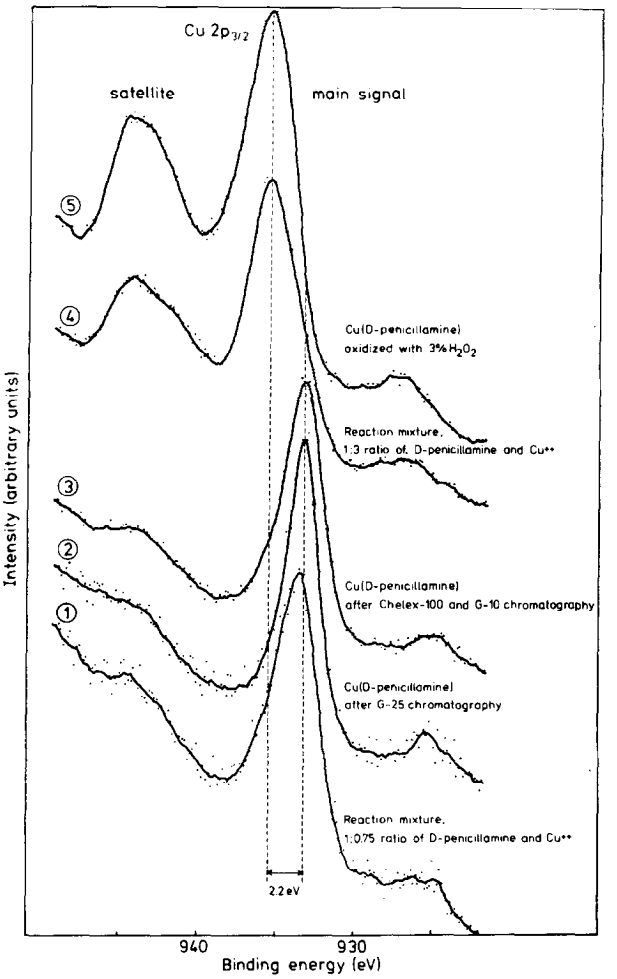

Figure 1: X-ray photoelectron spectra of the Cu $2p_{3/2}$ energy level. Recording conditions: work function 6.0 eV; analyzer energy 100 eV; sweep width 30 eV; sweep time 20 sec; number of channels 200; presure range 1-2 μTorr; number of scans 20, ①; 10, ②; 13, ③; 13, ④; 11, ⑤. The constant satellite near 925 eV is attributable to the Mg $K_{\alpha 3,4}$ X-ray contribution.

$$RS^- + Cu(II) \rightleftharpoons RS^-Cu(II) \rightleftharpoons \frac{1}{n}\left[RSCu(I)\right]_n \qquad (e)$$

After chromatography on an unbuffered Sephadex G-25 column considerable amounts of the remaining Cu(II) have disappeared (Fig.1, ②). It is interesting to note that even Chelex-100 (sodium form) does not remove copper from the complex as shown by the spectrum of a sample which has been treated with Chelex-100, desalted on Sephadex G-10, again treated with

Chelex-100 and rechromatographed on Sephadex G-10 (Fig.1, ③). In order to prove that photoreduction of Cu(II) during the measurement can be excluded excessive Cu(II) was added in a ratio of 3:1 to D-penicillamine (Fig.1, ④). The brown-green reaction mixture measured under identical conditions shows, as expected, a satellite specific for Cu(II) in the Cu $2p_{3/2}$ energy region and a shift by 2.2 eV to higher binding energies. Deep-violet species with an absorption maximum at 520 nm have been observed as short-lived intermediates in the reaction of many thiol-containing compounds with Cu(II) (25,26). It is highly attractive to assume that the violet compound is an intermediate species in the oxidation of thiol to the corresponding disulphide by Cu(II) (reaction (f)).

$$\frac{1}{n}\left[\mathrm{RSCu(I)}\right]_n \rightleftharpoons (\mathrm{RSSR})_{1/2}\mathrm{Cu(I)} \rightleftharpoons \frac{1}{2}\,\mathrm{RSSR} + \mathrm{Cu(I)} \qquad (f)$$

The circular dichroism spectrum of copper(D-penicillamine) after Sephadex G-25 chromatography shows negative Cotton effects at 565 nm, 408 nm, 310 nm and a positive Cotton band at 480 nm. A different spectrum was obtained for the reaction mixture of D-penicillamine and Cu(II) in the ratio 1:0.71 (5). The extraordinary stability of violet copper(D-penicillamine) may be attributed to the steric hindrance of the thiol group imparted by the methyl groups at the ß-carbon atom. It is noteworthy in this respect that also albumin bound copper is mobilized by D-penicillamine as a violet copper(D-penicillamine) complex (27). The complex is slowly attacked by 3% H_2O_2 (30 minutes, 25°C). In the X-ray photoelectron spectrum of the resulting brown precipitate a strong satellite (944.2 eV) and a shift of 2.2 eV of the Cu $2p_{3/2}$ main signal was seen (Fig.1, ⑤).

$$\frac{1}{n}\left[\mathrm{RSCu(I)}\right]_n + A_{ox} \rightleftharpoons (\mathrm{RSSR})_{1/2}\mathrm{Cu(II)} + A_{red} \qquad (g)$$

The spectrum of the sulphur $2p_{1/2,3/2}$ region demonstrates that the oxidation with H_2O_2 (reaction (g)) yields also sulphur atoms in a higher oxidation state (169.2 eV). Reducing agents, such as sodium dithionite, readily decolourize the violet complex. This reaction may be described:

$$\frac{1}{n}\left[\mathrm{RSCu(I)}\right]_n + A_{red} \rightleftharpoons \mathrm{RS^-Cu(I)} + A_{ox} \qquad (h)$$

These oxidation and reduction reactions of the violet copper(D-penicillamine) complex are probably less important in biological systems as

shown by the biological experiments of Wright and Frieden (16). Upon prior intravenous infusions of violet copper(D-penicillamine) the complex could be isolated from the urine of rabbits in a yield of up to 39%.

DISCUSSION

The nature of the basic disturbance of copper metabolism in Wilson's disease is still not known. Among the possible pathogenic mechanisms (28 29) the speculation that the newborn infant fails to convert its copper metabolism to normal deserves further consideration (30). According to this theory the abnormally continuing synthesis of a mitochondrocuprein-type protein in the liver is closely related with the primary defect. The high binding capacity and the unusual oxidation state of bound copper may result in a severe disturbance of the copper homeostasis leading to a reduced availability of copper for incorporation in apo-ceruloplasmin (31) and for biliary excretion. Further accumulation of copper may give rise to serious damage of the liver and to an overflow of copper to other tissues causing the well known clinical symptoms. D-penicillamine can form stable complexes both with Cu(I) and Cu(II). This versatility in the chelation of copper ions can account for the marked capability to reduce the tissue stores of copper in all stages of the illness, whereas the chelators EDTA, triethylenetetramine dihydrochloride and diglycyl-L-histidine bind exclusively Cu(II) (2,32). The primary attack of copper mobilization by D-penicillamine will depend on the onset of therapy. In the later stages of untreated Wilson's disease (33) the'reductive chelation' of Cu(II) bound to a great number of proteins and the subsequent urinary excretion as a stable chelate will be most important. Beside copper mobilization D-penicillamine may play an important role in maintaining the sulphhydryl-disuphide equilibrium, thus, stabilizing the oxidation-reduction potential in the cell.

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