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Copper Mobilization by the Proteins at Various pH Values

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Abstract

The paper investigates reaction of certain relevant proteins with copper ions in response to changes in pH. The effect of pH on copper mobilization by proteins in vitro conditions and serum copper/iron ratio as a function of pH as well as its connection with pathological alteration are followed. This experiment showed that proteins mobilize copper ions by different mechanisms depending on pH. The possible relationship between Alzheimer's disease, Wilson's disease, prion diseases, cirrhosis, Down syndrome and pH-dependent copper-protein complex is also considered.

Keywords: Copper-protein complex; Copper mobilization; pH; Alzheimer's disease; Wilson's disease

Introduction

Much evidence has been brought so far for the relationship between the level of copper within the body fluids and certain patho-physiological phenomena [1–9]. Thus, a good correlation between non-ceruloplasmin-bound copper and 24 h urinary copper excretion associated with renal tubular acidosis in Wilson's disease was reported [1]. Serum copper and ceruloplasmin in Wilson's disease are significantly lower as compared to normals [2]. In the meantime, a copper-binding protein in childhood cirrhosis was identified as a significant factor in hepatic intracellular copper accumulation [3]. Cancer patients also show significantly reduced serum copper complex protein values as compared to normals individuals and non-cancer patients [4]. On the contrary, serum copper and ceruloplasmin are higher in the patients with cutaneous leithmaniasis than those of controls, whereas Se, Zn, Fe, and transferin are lower [5]. The level of plasma Cu in patients with rheumatoid arthritis is also significantly increased as compared to normal individuals [6]. High concentrations of copper are found in the vicinity of A_β amyloid deposits in Alzheimer's disease (AD) [7]. Cu^{2+} also causes the peptide to aggregate to a great extent and markedly potentiates the neurotoxicity exhibited by A_β in cell culture [8]. Copper-selective chelators have been shown to dissolve A_β deposits extracted from AD-post-mortem brain specimens [9]. In addition, serum copper/zinc ratio and ceruloplasmin are higher in elderly than in healthy adults due to high copper values, whereas in the disabled, both high copper and low serum concentration are present [10].

Such findings represent just a few examples suggesting the relationship between the ability of proteins to react with copper ions liberated from their precipitates, copper level in the fluids of the body and various diseases.

Therefore, this paper aims at investigating the reaction between certain relevant proteins and copper ions in response to changes in pH. In addition, the main characteristics of this reaction and, of course, the differences from the well-known biuret reaction are investigated. The relationship between copper level and that of the iron across the physiological pH range, as well as a possible connection between these parameters and the pathological alteration are also followed.

Materials and Methods

Apparatus

A Carl Zeiss Spekol spectrophotometer with 1 cm matched cells was used for all spectral measurements. A laboratory centrifuge and calibrated glassware were also used.

Chemicals

All reagents used in this study were of analytical reagent grade and all solutions were prepared with twice distilled water. Chemicals were from Sigma unless stated otherwise. Ninhydrin, copper carbonate basic, copper sulfate, sodium phosphate tribasic dodecahydrate, and potassium ferrocyanide trihydrate were purchased from Merck. Copper phosphate and copper ferrocyanide were obtained by the reaction of copper sulfate with sodium phosphate tribasic dodecahydrate and copper sulfate with potassium ferrocyanide, respectively. The resulted precipitates were washed a few times with twice distilled water, centrifuged and dried.

Protein solutions with pH 7 - 13. Peptone from meat 10% in water was used. Each sample of 10 ml protein solution was treated with a 40% solution of KOH to reach a pH value between 7 and 13.

Protein solutions with pH 6.5 – 8.0. Volumes of 4 ml of 0.1 M phosphate buffer solution with pH 6.5 – 8.0 was added to each 1 ml of 10% solution of peptone.

Protein solutions with pH 6.5 – 10.0. Volumes of 2.5 ml of 10% solution of peptone were diluted with 7.5 ml water and treated with 0 – 0.1 ml of 5 M solution of KOH.

Biological materials

Human serum was obtained from the University Polyclinic of Iasi being drawn from healthy subjects. Corrections of pH (pH 6.7 – 8.5) were made with concentrated sulfuric acid and KOH solution, respectively.

Procedure

The effect of pH on copper mobilization by proteins under *in vitro* conditions was investigated within the following experiments.

1. 1 mL of 10% protein solution, pH 7 – 13, 4 ml of water and 100 mg copper salt were mixed by stirring for 15 min and then centrifuged. The biuret absorbance was measured at 540 nm in 0.5 cm glass cuvette and copper concentration of the supernatant determined.
2. Protein solutions (5 mL, pH 6.5 – 8.0) were stirred with copper ferrocyanide for 15 min (100 mg salt/5 mL of solution) and centrifuged. Then, copper concentration of the supernatant was measured. Separately, before adding buffered solution with pH 6.5 – 8.0, volumes of 5 ml protein solution were treated with 0.1 mL of $FeSO_4$ solutions containing

0.1 mg mL^{-1} Fe^{2+}. The mixtures were stirred and centrifuged. The iron concentration of each supernatant was measured.

3. The above-described experiment was also made with human serum with pH 6.7 – 8.5.
4. Volumes of 10 ml protein solutions with pH 6.5 – 10.0 were treated with 100 mg powder of copper phosphate, stirred for 2 hours, and centrifuged. The absorbance at 540 nm of the supernatants was measured against water. A blank without copper phosphate was also carried out. Then, 1 ml of 5 M solution of potassium hydroxide was added into each test tube and the absorbance was read again.

Analysis

Iron was assayed with bathophenanthroline [11, 12]. Copper was determined colorimetrically with bathocuproine sulphonate after appropriate dilutions of the samples [13, 14]. The protein content was determined by the Lowry procedure [15] and by another method using a ninhydrin reagent [16].

Statistical analyses

The standard deviation (*S*), standard deviation of the mean (s_x), the correlation coefficient, r, and *t* parameter, as well as the coefficient of variation (CV %) were calculated. Means were compared with Student's t test.

Results

1. The absorbance of the copper-protein complex was proportional to the copper amount extracted by the proteins (Fig. 1). Various copper precipitates caused different degrees of copper mobilization. Figure 1 also showed that copper was mobilized by the proteins even at lower pH values. Thus, between pH 7.0 and pH 9.0, the rate of copper mobilization quickly increased depending on pH and the type of copper salt. The next range of pH, from 9.0 to 11.5, was characterized by a slower increase in the absorbance as a function of pH. The correlation coefficient, r, confirmed that the amount of copper mobilized by proteins was acutely sensitive to changes in pH.

2. The proteins mobilized copper ions even at a physiological pH ranging from 6.5 to 7.5. Thus, peptone solution had a copper concentration of 49 μg ml^{-1} Cu^{2+} at pH 6.5 and of 238 μg ml^{-1} Cu^{2+} at pH 7.5 (Fig. 2). A decrease in copper extraction was observed when pH was lowered from the optimum pH of 7.38 to 6.8. At the same time, the iron ions have shown the tendency to precipitate as phosphoric salts at the small pH-values and as $Fe(OH)_3$ in the more alkaline solutions. Thus the highest iron level *in vitro* would be observable at a pH close to the optimum determined *in vivo*. A concentration of 140 – 152 μg Fe^{2+} in 100 mL of protein solution was found at pH 7.4. It decreased to 58 μg Fe^{2+} in 100 mL at pH 8.0 and 26 μg Fe^{2+} in 100 mL at pH 6.5.

3. Similarly, the higher the pH the bigger the amount of serum copper, while, serum iron presented a maximum at the physiological pH (not shown). Serum Fe^{2+}/Cu^{2+} ratio also showed a complex dependence on pH, which was predictable from the individual concentrations of Fe^{2+} and Cu^{2+}.

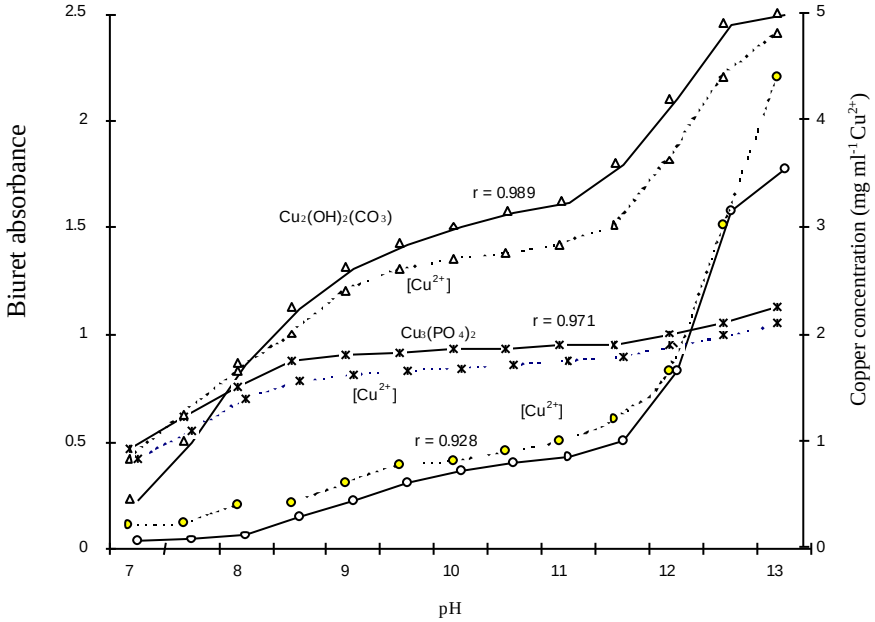

Figure 1. Copper ions mobilization from precipitates by the peptone solutions at various pH values (Biuret absorbance from -o- $Cu_2[Fe(CN)_6]$, -x- $Cu_3(PO_4)_2$, -Δ- $Cu_2(OH)_2CO_3$.

4. The treatment of the protein solution with the precipitate of copper phosphate caused the mobilization of Cu^{2+} as a function of pH (**Figure 3**). The absorbance of the mixture increased accordingly. Adding the alkaline solution resulted in a constant increase in the biuret absorbance by 0.300. This experiment showed that proteins react with copper ions by different mechanisms as a function of pH. Thus, they mobilized copper ions by binding them partly as a biuret complex, partly by another mechanism at low pH (17,18). Proteins bind copper at higher pH values to afford the biuret complex.

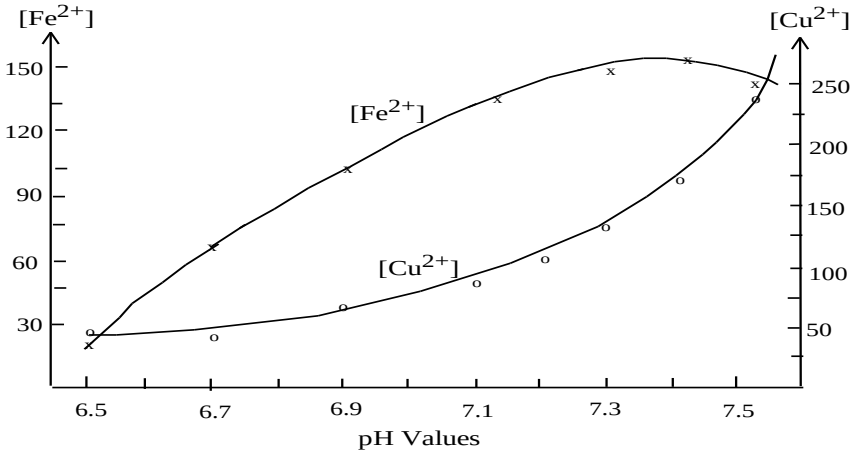

Fig. 2. The dependence of serum Cu^{2+} and Fe^{2+} concentration, expressed as μg/100 ml, on the pH values (-o- = $[Cu^{2+}]$, and -x- = $[Fe^{2+}]$)

Figure 2. The dpendence of serum Cu^{2+} and Fe^{2+} concentration, expressed as μg/100ml, on the pH values (-o- =[Cu^{2+}] and -x- = [Fe^{2+}].

Discussion

There is a strong relationship between copper ions, proteins and pH, which have to be taken into consideration for clinical purposes. Proteins mobilize copper ions depending on pH and serum copper/iron ratio may be affected also by pH. Also, pH changes could drastically affect the relationship between the other serum ions, such as Zn^{2+}, Mn^{2+}, Ca^{2+}, Se^{2-}, etc.

Structure

Copper (II) – peptide binding begins with the coordination of the Cu^{2+} aqua ion to an amino or an imidazole nitrogen that serves as an anchor site to initiate metal chelation through succesive deprotonation of peptide bonds as the pH raised. The ε-terminal amino group of lysine residue, which binds copper in the relatively unstable ML complex, may play this initiating role. Then, the deprotonation of the amide group at the Lys-Ser junction occurs, and, finally, because of the important steric crowding around the metal ion, another complex forms via the deprotonation of the hydroxyl group of the serine residue [17]. Also, a major contribution to the stability is exerted by non-bonding side chains of arginine and tyrosine. The effect is explained on the basis of spectroscopic data by the formation of a secondary fence schielding the Cu(II) binding site from the bulk of the solution. [18]. When examining the structure of Cu(II)-complexes and ligating atoms in copper proteins and in some model systems [19], the parameters of ERS typical for blue copper ions are not observed in copper proteins and ferments. Such a structure is of possible importance for the understanding of interactions of copper ions with proteins as a function of pH.

Cirrhosis

The copper content in cirrhosis liver supernatant is found to be about 9-fold higher than in the control liver supernatant. A copper-binding protein is solely responsible for binding about 35 % of the total supernatant copper in childhood cirrhosis [3]. Hypercupriuria is associated with renal tubular acidosis suggesting a low binding capacity of serum proteins for copper. The amino acid composition of this protein revealed the presence of aromatic amino acids and higher content of glutamic acid and aspartic acid followed by glycine and serine. The ratio of basic amino acids strongly indicates that it is an acidic protein. The cysteine content in this protein is insignificant, which further corroborates the possibility that acidic amino acids might be prominent candidates for binding copper. Thus, the 50-kDa MCuBP apparently makes a major contribution to the total copper binding activity in cirrhosis liver cytosol and may play a significant role in hepatic intracellular copper accumulation [3].

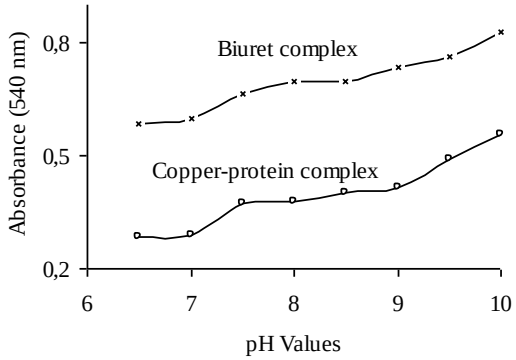

Figure 3. The effect of pH on the formation of copper-protein complex.

Alzheimer's disease

The relationship between copper, protein and pH should now examined in the context of potential therapeutic intervention in AD, Wilson's disease, cancer, rheumatoid arthritis, cirrhosis, etc. Thus, the cerebral cortex of Alzheimer's and Down syndrome patients is characterized by the presence of intracellular and extracellular deposits of proteins in tangles, neuropil threads, and neuritic plaques are correlated with neuronal dysfunction leading to dementia [20]. For these cases, a more general mechanism must exist, ultimately leading to neuronal degeneration. The genes encoding ß amyloid precursor protein (βAPP) and ubiquitin-ß (UBI-ß) protein each contain several GAGAG motifs. Fred van Leeuwen and his colleagues theorized that the mistake arose during protein synthesis and such mistakes may help cause Alzheimer's in the majority of patients [21]. Thus, during aging, single neurons may generate and accumulate abnormal proteins, consequently leading to cellular disturbances and causing degeneration. Also, Cu(II) is reduced by A_β peptides in Alzheimer's disease and Cu (I) mediates O_2-dependent cell-free H_2O_2 generation, and these properties are directly correlated with the Cu(II)-mediated potentiation of A_β neurotoxicity in cell culture. It was concluded that neurotoxicity was mediated via peptide-Cu(II) interactions rather than the effect of free Cu(II) upon the cell. Cu(II) interaction with A_β may be exaggerated in AD where copper levels are abnormally elevated. Therefore, the abnormally high concentrations of copper in AD neuropil and in amyloid may reflect a pathogenic neurochemical milieu that both aggregates A_β and induces H_2O_2 production [7]. Since most of the added Cu(II) would be bound by the peptide within the culture medium, it was concluded that neurotoxicity was mediated via peptide-Cu(II) interactions rather than the effect of free Cu(II) upon the cells. The neurotoxicity of A_β in cell culture has been linked to H_2O_2 generation by an unknown mechanism [7].

These data suggest that formation of an A_β-copper complex may be a path-physiological interaction, and a new target for therapeutic interdiction in AD [7]. These findings suggest that certain metal ions may be important in exacerbating and perhaps facilitating A_β-mediated oxidative damage in Alzheimer's disease. Therefore, these metal ions may be important cofactors in A_β-associated oxidative damage.

Wilson's disease

Wilson's disease is an autosomal recessive disorder of copper accumulation in various organs, with most common clinical manifestations such as hepatic, neurologic, and renal disfunctions. Over 60 specific mutations of ATP7B, the gene for Wilson's disease, have been reported in patients. The ATP7B protein is a copper transporter involved in the intracellular

trafficking of copper in hepatocytes [22]. Serum copper and ceruloplasmin in Wilson's disease are significantly lower as compared to normals, controls, and relatives of Wilson's disease patients, whereas marked hypercupriuria was observed in Wilson's children only. These patients of Wilson's disease were confirmed by measuring liver biopsy copper, which was about nine times higher than normal hepatic copper content. Over 90% of the copper found in the plasma is bound to ceruloplasmin, the remaining 60-120 μg/L is attached mostly to albumin and some to histidine or glutamine. In 95% of the Wilson's patients the serum ceruloplasmin levels are well below the normal values [2]. In some cases the copper protein is virtually absent. The accumulated copper exceeds the storage capability of the liver, spills into bloodstream, and precipitates in suitable environments including the brain, the kidneys, and the eyes. The metal content of the deposits can be quite high (up to 1%). Lack of ceruloplasmin as the principal cause of copper accumulation falls short of explaining one important aspect of Wilson's disease: about 5% of the patients have normal levels of the blue protein [2].

Prion diseases

Prion diseases result from conformational alteration of PrP^{c}, a cell surface glycoprotein expressed in brain, spinal cord, and several peripheral tissues, into PrP^{Sc}, a protease-resistant isoform that is the principal component of infections prion particles. Several lines of evidence have recently suggested the possibility that PrP^{c} may play a role in the metabolism of copper. It was reported that copper rapidly and reversibly stimulates endocytosis of PrP^{c} from the cell surface. PrP^{c} could serve as a recycling receptor for uptake of copper ions from the extracellular milieu [23].

Consequences for therapy

At least in the case of Alzheimer's disease, these experimental data suggest the opportunity to use D-penicillamine to prevent the formation of neuritic plaques or to destroy the old ones. In addition, the role of free copper ions in accumulating abnormal proteins or in A_{β} neurotoxicity must be investigated. Also, the relationship between the copper or iron concentrations in the body fluids and the small changes in pH must be more investigated.

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