The Presence of Zinc in Human Cytocuprein and Some Properties of the Apoprotein

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SUMMARY

Purified cytocuprein from human liver, brain, and erythrocytes was found to contain near 2 g atoms of zinc per mole of protein. Both the copper and zinc were strongly bound and only relatively small amounts of them could be removed from the protein by dialysis against EDTA and 1,10-phenanthroline under various conditions. Virtually all of the metal was removed by treatment with potassium cyanide at neutral pH. There appeared to be very little change in the tertiary structure of the protein upon removal of the metal. This was indicated by the observations that the velocity and equilibrium sedimentation properties, the immunological properties, and absorption in the 200 to 240 m\(\mu\) range of the native and apoprotein are similar. However, such treatment resulted in a diminution in the intensity of absorption of cytocuprein between 240 and 400 m\(\mu\) and the disappearance of the broad absorption peak with a maximum near 675 m\(\mu\).

The copper-containing proteins known as erythrocuprein, hepatocuprein, and cerebrocuprein have been shown to be identical and the name cytocuprein was proposed for them (1). We have recently found that cytocuprein also contains zinc. The first indication of this was obtained in studies of \(^{64}\)Zn exchange by the carbonic anhydrase isoenzymes in hemolysates (2). In addition to these enzymes, radioactivity was found to be associated with a protein identified as cytocuprein. This result was thought to be caused by a replacement of the copper in the protein by zinc. However, an attempt to remove copper under the conditions used for the zinc exchange reactions was unsuccessful and our attention was directed to the possibility that the native protein actually contained the latter metal. Detailed analyses conducted on cytocuprein from brain, liver, and erythrocytes have shown that the molar levels of zinc and copper in the protein are nearly equal.

An understanding of the roles of zinc and copper in the physical-chemical and biochemical properties of cytocuprein is dependent upon the complete removal of the metals without inducing denaturative changes in the protein. Conditions for the preparation of an apoprotein were determined in order to provide suitable material for such future studies. The properties of the apocytocuprein indicate that no significant changes in tertiary structure occur upon removal of the metal.

METHODS

Cytocuprein was isolated from human erythrocytes, liver, and brain by methods described previously (1, 3). All buffers were prepared with water which had been passed through a mixed bed ion exchange resin (Continental Deionized Water Service, Chicago, Illinois). Water used in metal analyses was distilled twice in a quartz apparatus. Protein samples to be analyzed were dialyzed near 4\(^\circ\) for at least 48 hours against 0.15 m NaCl which had been passed over a column of Dowex A-1 chelating resin (Dow Chemical Company, Midland, Michigan).

Copper analyses were performed by the method of Van de Bogart and Beinert (4). A modification of the dithizone method described by Malmstrom (5) was used to assay zine. The modifications included wet ashing of the protein (4) and the use of volumes of reagents sufficiently small so that all steps of the procedure through the extraction of the zinc into the organic phase could be carried out in 15-ml Pyrex test tubes. Since the copper of cytocuprein was a potential source of interference in the measurement of zinc, control assays were conducted with copper at levels near those present in the protein samples.

Nitrogen analyses were carried out by a micro Kjeldahl procedure (6), and cytocuprein concentrations are based on a nitrogen content of 16.9% (7). Quantitative precipitin reactions were carried out in a 3.0-ml system as described by Cohn, Wetter, and Deutsch (8). Precipitin reactions in gels utilized the Ouchterlony technique (9). Absorption spectra were measured manually with a Beckman DU-2 spectrophotometer.

Sedimentation analyses employed a Spinco model E ultracentrifuge equipped with both schlieren optics and a photoelectric scanning system (10). A wave length of 265 m\(\mu\) was generally used with the latter optics.

RESULTS

The levels of zinc and copper in cytocuprein preparations from liver, brain, and erythrocytes are presented in Table I. The experimental variations encountered in the zinc assay are included since the precision of these measurements was not as high as that for the copper assay. The molar levels of the two metals are similar although the values for zinc are generally lower than those for copper. The differences are less than 1 S.D. except for cytocuprein from liver. In this case the levels of both
Results of studies on chelation of copper and zinc of cytocuprein

The copper and zinc contents before treatment with chelating agents were 1.98 and 1.92 g atoms per mole of protein, respectively.

| Condition of chelation | pH | Duration of dialysis | Temperature | Copper | Zinc |
|------------------------|----|----------------------|-------------|--------|------|
| 0.1 M/l,1O-phenanthroline | 7.4 | 72 | 4° | 2.01 | 1.91 |
| 0.1 M/l,1O-phenanthroline | 5.5 | 72 | 4° | 1.98 | 1.67 |
| 0.1 M/l,1O-phenanthroline | 5.5 | 24 | 27 | 1.63 | 1.69 |
| 0.2 M/l,1O-phenanthroline + 10° M EDTA | 7.4 | 96 | 4° | 1.96 | 1.73 |
| 0.2 M/l,1O-phenanthroline + 10° M EDTA + 10° M ascorbate | 7.4 | 96 | 4° | 1.96 | 2.00 |
| 0.05 M Tris-HCl + 0.05 M KCN | 8.0 | 72 | 27 | <0.01 | <0.05 |

1 These analyses were performed by personnel of the Nuclear Engineering Laboratory of the University of Wisconsin.

2 Expressed as gram atoms per 33,600 g of protein. Values in parentheses are the number of analyses conducted.

3 Experimental variations given are 1 S.D.
The results of a starch gel electrophoretic experiment on cytocuprein and on the apoprotein are shown in Fig. 4. It is apparent that the major component of the apoprotein has a much higher anodic mobility than the parent protein. The cytocuprein used in this experiment had been stored for over 3 months at −20°C and had been thawed and refrozen numerous times during this period. This apparently leads to the generation of several additional minor and more anodic components than noted in the fresh material (1). The liability of cytocuprein to form more anodic components has been previously noted (3, 13). The less anodic of the numerous minor components noted in the apocytoprein preparation appear to have similar electrophoretic mobilities to the more anodic minor components of the native protein. It can be seen from Fig. 4 that the major portion of the apoprotein is composed of two electrophoretic components which appear to be analogous to the two cytocuprein components noted in freshly prepared material (3, 13).

The absorption spectra of the visible and ultraviolet regions for cytocuprein and the apoprotein are presented in Fig. 5. The apoprotein had a marked decrease in the intensity of absorption in the 250 to 290 nm range and the broad peak with the maximum at 675 nm was absent. No change in the absorption of the two proteins over the 200 to 240 nm range is evident.

**DISCUSSION**

The zinc in cytocuprein appears to be more firmly bound than that of carbonic anhydrase. Lindskog and Malmstrom (14) were able to remove all of the zinc from carbonic anhydrase by dialysis at pH 5.0 against 10⁻² M 1,10-phenanthroline for 2 to 7 days. Dialysis of cytocuprein against this chelating agent at pH 5.5 for 3 days removed only a small amount of this metal. These chelating experiments removed zinc more readily than copper, but both metals were strongly bound. The labeling of cytocuprein with ⁶⁵Zn under these conditions as observed earlier (2) appears to have resulted from the exchange of a relatively small portion of the zinc.

The observation by Markowitz et al. (11) that only part of the copper of cytocuprein can be removed by dialysis against cyanide at 3–5°C was confirmed by our experiments. However, at room temperature the zinc and copper were completely removed in 72 hours. The apoprotein has good solubility properties unlike material produced in earlier experiments (15).

The $S_{20,w}$ value of cytocuprein was not affected significantly by the removal of the metals, whereas the removal of copper from ceruloplasmin results in a marked decrease in the $S_{20,w}$ value and an increase in the axial ratio (16). The apocytoprein preparations contained small but variable amounts of protein which sedimented more slowly than the 2.86 S component. Studies on cytocuprein which had been subjected to mild performic acid oxidation or to reduction and alkylation have indicated that the protein contains a subunit of molecular weight near 12,000 (13). It is possible that the cyanide has also produced a small amount of a similar subunit through its known action of cleaving disulfide bonds. However, the production of such material by cyanide
appears limited since the major component had the same molecular weight as the native protein.

The intensity of the absorption spectrum in the visible 240 to 350 m\(\mu\) region for apocytocuprein is considerably less than the native protein and the peak with the maximum at 675 m\(\mu\) is lost. These changes are caused by the removal of copper and possibly zinc. The reason for the decreased absorption by the apoprotein in the region from 250 to 290 m\(\mu\) is not clear but may relate to metal ligands, changes in tertiary structure, or both, following removal of the metals.

Changes in peptide structures from a helical to a non- or less helical structure have been shown by Imahori and Tanaka (17) to be accompanied by a large increase in ultraviolet absorption in the region of 190 m\(\mu\). Rosenheck and Doty (18) have shown that it is feasible to draw inferences from ultraviolet absorption data as to the extent of helical structure in peptides, and Edsall (19) has presented such data for several proteins. A specific example is the marked decrease in absorption in the 180 to 235 m\(\mu\) region by human carbonic anhydrase B following its conversion to a more helical structure upon acid denaturation (20).

A similar effect has been noted for human cytocuprein when its axial ratio is decreased by treatment with ethanol-chloroform (15) under conditions simulating the Tsuchihashi (21) precipitation denaturation of hemoglobin. Removal of metals from cytocuprein did not effect the absorption between 190 and 235 m\(\mu\) which indicates that no gross changes in tertiary structure and symmetry results from such treatment. The nondenaturative removal of the zinc from bovine carbonic anhydrase B has been shown to effect no changes in the optical rotary dispersion properties of this enzyme (14).

The observation that cytocuprein contains equimolar levels of copper and zinc suggests that there might be some interaction between these metals. Chelation experiments discussed above show that at least part of the zinc can be removed without loss of copper. However, studies of reconstitution of the apoprotein appear more attractive at present than attempts to remove one of the metals selectively. Such studies will permit the evaluation of the role of each of the metals on the light absorption properties, electron paramagnetic resonance spectrum, and immunological and biochemical properties.

Although the presence of cytocuprein in liver, brain, and erythrocytes has been recognized for many years, a biochemical role for the protein was not suggested until recently. McCord and Fridovich (22) have reported that cytocupreins from bovine and human erythrocytes possess superoxide dismutase activity, but the physiological significance of this activity is uncertain. Probably the zinc in cytocuprein is related to this biochemical function. We have previously suggested the name cytocuprein for the protein which was known as either erythrocuprein, hepatocuprein, or cerebrocuprein, depending on the tissue source (1). In view of the zinc content of this protein, the name cytocuprein is not accurately descriptive. A more appropriate name should relate to the biochemical function of the protein when it becomes known.

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