PURIFICATION OF LOW MOLECULAR WEIGHT COPPER PROTEINS FROM COPPER LOADED LIVER

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SUMMARY

Purification of low molecular weight copper binding proteins from the livers of copper loaded male rats was achieved by sequential ultracentrifugation (186,000g, 2h), ultrafiltration (Amicon PM 30), gel filtration (Sephadex G-75) and anion exchange chromatography (DEAE - Biogel A) of soluble tissue extracts. The three major copper-associated polypeptides obtained which had molecular weights of about 7000, 9,000, and 12,000 daltons contained approximately 2.5g atoms of copper per mole. Amino acid analyses indicated a similarity between these proteins and the copper protein 'L-6D' isolated earlier from livers of Wilson's disease patients and distinguished them from metallothioneins which have been isolated from animals administered other trace metal ions.

Heavy metals including copper have been detected in tissues of several species in association with a low molecular weight protein which Vallee first described and termed metallothionein (1-5). Administration of cadmium or zinc salts substantially increase the amount of this protein in both kidney and liver (6-16). It is not yet clear whether mercury has a similar effect (17,18).

Although copper is also known to be present in metallothionein, changes in tissue levels of this protein on administration of copper have not been demonstrated. However, in some instances, characterization of partially purified low molecular weight copper proteins has suggested that there is a larger amount of protein but that this may not be due to enhanced synthesis of a metallothionein. For example, Evans has demonstrated very recently that a small soluble protein from copper loaded rats did not exhibit the high cysteine content of metallothionein (19). Rajagopalan and colleagues have reported in abstract form that a protein different from metallothionein appeared on injec-

tion of copper (20). Earlier, Bloomer and Sourkes (21) had described a mixture of soluble proteins from copper loaded rats which did not appear to contain metallothionein. The present communication reports that on further purification three major copper containing polypeptides can be obtained, and that if metallothionein is present among the low molecular weight copper proteins of copper loaded liver it is certainly only as a minor component.

METHODS

Male Wistar rats (300-400g) after subcutaneous injection for 6 consecutive days with $CuSO_4$ (2 mg Cu^{++}/kg) were sacrificed by decapitation. Portions of the excised livers were used for determination of total tissue copper by a modification of the method of Eden and Green (22). A 20% homogenate of the remainder in 0.05 M Na H_2PO_4, pH 7.0 was prepared in a Waring blender (5 x 15 sec). After centrifugation at 16,300g for 30 min. to remove large particulate material, the supernatant was spun for 2h at 186,000g. This supernatant was passed through an Amicon PM 30 filter, thereby removing material larger than 30,000 daltons, and concentrated by a second ultrafiltration with an Amicon UM 2 filter. The concentrate was applied to a Sephadex G-75 column (2.5 x 90 cm.). 5 ml fractions were monitored for absorbance at 280 and 250 nm and copper concentration (Pye-Unicam SP 1800 Atomic Absorption Spectrometer). Fractions comprising the low molecular weight copper peak were pooled and concentrated either by ultrafiltration (Amicon UM 2) or dialysis and lyophilization. This material was applied to a column of DEAE-Biogel A (2.5 x 20 cm) with elution as indicated in the figure legend. Fractions were monitored and copper containing peak fractions were concentrated by either of the methods mentioned above.

Protein concentrations were determined by the fluorometric procedure of Böhlen et al (23) which accurately detected less than 1 µg of protein. This procedure proved a major advantage over other commonly used methods of protein determination since neither the copper binding proteins nor the metallothioneins contain significant amounts of aromatic amino acids. Polyacrylamide gel electrophoresis was performed with discs or slabs of 10% acrylamide in (a) 0.1 M glycine-acetic acid, pH 3.6; (b) 0.1 M Tris-glycine, pH 8.9 or (c) 0.1 M Tris-HCL, pH 7.4 containing 1% Na dodecyl sulfate. Molecular weights were estimated by interpolation from plots of log molecular weight versus distance migrated in the latter system by the following proteins as standards having the subunit molecular weights indicated: chymotrypsinogen (25,000), hemoglobin (16,750), lysozyme (14,000), cytochrome C (12,000) and insulin (5,734). $^{67}Cu^{++}$ was prepared (24) by irradiation of natural zinc with Bremsstrahlung from the linear accelerator at the University of Toronto and counted in a well-type gamma spectrometer. Amino acid analyses were performed employing a Technicon TSM analyser after hydrolysis in 6 N HCl *in vacuo* for 20h at 110°C.

RESULTS

When the livers of rats were loaded with copper to a level of about 200 µg per g. wet tissue, the amount of the metal ion at the various stages of isolation of low molecular weight copper proteins was as shown in table I. The 40% of the

Table I

Distribution of copper during isolation of low molecular weight proteins from copper-loaded liver.

Fraction	Cu (μg)	% of total
Total liver homogenate	9400	100.0
186,000g supernatant	5600	60.0
PM 30 concentrate	5250	55.0
PM 30 ultrafiltrate	176	2.0
UM 2 concentrate	69	0.7
UM 2 ultrafiltrate	10	0.1
Sephadex G-75 peak	40	0.4

Copper in the homogenate was determined chemically (22) and in the other fractions by atomic absorption spectrometry.

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total liver copper which was removed after high speed centrifugation presumably was associated mainly with particulate subcellular fractions. By far the greatest proportion of the copper remaining in the soluble supernatant was retained by an Amicon PM 30 ultrafilter and, therefore, must have been associated with material having a molecular size greater than the equivalent of 30,000 daltons. Of the 2% of the total copper in association with smaller molecules about 1/3 (or 0.7% of the total) was retained by an Amicon UM 2 ultrafilter. About 1/2 of this (or 0.4% of the total) was associated with the small copper proteins obtained by gel filtration on Sephadex G-75. When copper loaded animals were injected intravenously with $^{67}Cu^{++}$ 1/2 h. before sacrifice the percentages of the total liver radioactivity in the various fractions were the same as those listed in table I.

Fig 1 shows the G-75 elution profile obtained in a preparation where about four times more copper loaded liver was used as starting material as in the experi-

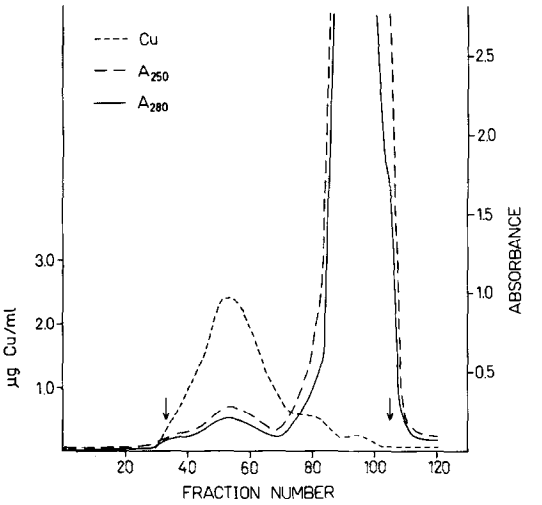

Figure 1: Gel filtration of ultrafiltrate (Amicon PM 30) of high speed supernatant from liver homogenate on a Sephadex G-75 column (2.5 x 90 cm) in 1 mM Tris-HCl, pH 8.6. 5 ml fractions were collected and monitored for absorbance at 2 wavelengths and copper content as indicated. The arrows indicate the positions of the void and bed volumes of the column.

ment represented in table I. Near the bed volume is a large peak of absorbance at 250 and 280 nm. These fractions are visibly yellow, non-proteinaceous and only small amounts of copper are present (in fractions 75-83 and 90 to 100). There is a principal copper peak centred at an elution volume of 250 ml. Over the major portion of the peak (fractions 42 to 69) the absorbance at 250 nm is greater than that at 280 nm. The fractions comprising the shoulder and the major portion of the peak were pooled separately and only the latter part processed through the subsequent anion exchange step. Analysis of the material from the major peak revealed a copper content of about 2.5 g atoms per mole (calculated on the basis of a mean molecular weight of 9000 daltons). In several other preparations from copper-loaded animals, values between 1 and 5 g atoms of copper per mole were obtained. Polyacrylamide gel electrophoresis (fig 2, gel B) showed the presence of three major polypeptide bands with molecular weights of approximately 12,000, 9,000 and 7,000 daltons.

Amino acid analyses yielded the composition shown in table II which also

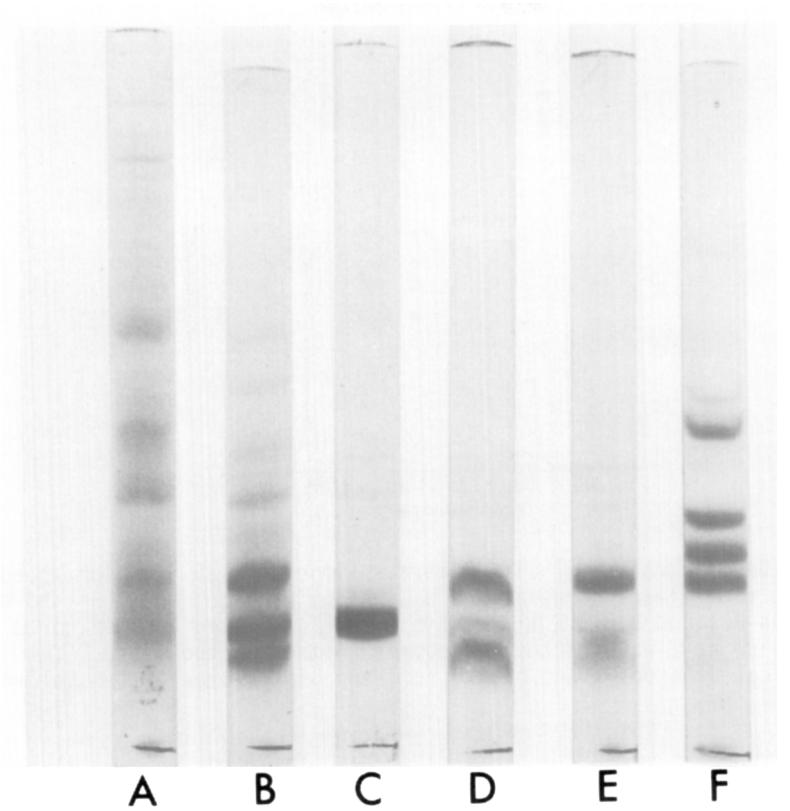

Figure 2: Protein staining (Coomassie blue) bands after polyacrylamide gel electrophoresis in system (c) of Methods. Samples were prepared by incubation in a boiling water bath for 2 min. in 1% SDS and 40 mM dithiothreitol. The following amounts of protein from the chromatography fractions indicated were applied: Gels A and B: 25 μg from fractions 30-41 and 42-69 of fig 1 respectively. Gels C,D,E: 25, 18, 17 μg from fractions 10-15, 16-40, 41-46 of fig 3 respectively. 10μg each of chymotrypsinogen, hemoglobin, lysozyme and cytochrome C were applied to gel F.

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includes for comparative purposes the composition recently reported by Evans (19) for a similar preparation from copper loaded rat liver as well as those of a smal copper protein isolated from the livers of a Wilson's disease patient by Morell et al (25). The amount of each of the amino acids in the three different preparations are very similar. Most notable are the cysteine values which are about four times less than that of the metallothioneins, the major low molecular weight proteins present after loading with other trace metals such as Cd^{++} and Zn^{++}.

The three major copper-associated polypeptides of our G-75 copper peak

Table II

Amino acid composition of low molecular copper proteins from liver.[a]

Amino acid	Cu-loaded rat liver		Liver of Wilson's disease patient[b]
	A	B	
Aspartic acid	10.2	11.4	8.8
Threonine	7.9	7.2	5.8
Serine	7.3	7.6	7.1
Glutamic acid	12.6	11.9	11.2
Proline	4.0	3.9	5.5
Glycine	10.1	8.8	8.6
Alanine	4.9	6.8	8.2
Valine	4.3	5.1	7.1
Half-cystine	7.5	5.0	4.3
Methionine	3.2	-	1.5
Isoleucine	5.0	4.3	4.9
Leucine	6.2	7.0	6.7
Tyrosine	2.0	-	1.1
Phenylalanine	3.7	-	3.1
Lysine	13.2	13.0	10.8
Histidine	1.7	-	1.5
Arginine	2.5	3.0	2.6

A proteins obtained from gel filtration step (fractions 42-69 of fig. 1)

B proteins obtained by Evans et al (19).

a expressed as residues per 100

b from Morell et al (25).

were further resolved on DEAE Biogel A (fig 3). The shallow salt gradient used for elution tended to cause smearing of the copper - containing proteins but was necessary for their resolution. The first small peak (fractions 10-15) contained exclusively the polypeptide weighing 9,000 dalton (fig 2, gel C), the broad copper peak (fractions 16-39) contained the polypeptides weighing 7,000 and 12,000 daltons (fig 2, gel D), whereas the very sharp copper peak (fractions 40-46) contained primarily the largest polypeptide. The copper contents of none of the individual polypeptides exceeded that of the three together. Their seeming similarity raises the possibility that they may be different forms of the same protein but gel electrophoresis in two non-dissociating systems (see Methods) also showed three narrowly separated bands having the same relative staining intensities as seen in the dissociating system (fig 2). Therefore, it seems highly unlikely that the three bands observed are a result of disruption of subunit interactions.

DISCUSSION

We have been able to purify three different small proteins with which

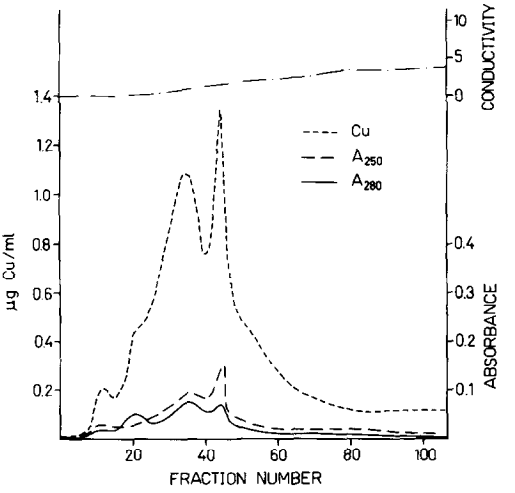

Figure 3: Anion exchange chromatography of pooled gel filtration fractions (42-69 of fig 1) on a DEAE Biogel A column (2.5 x 20 cm). Elution was with a salt gradient from 0 to 0.1 M NaCl which resulted in the conductivity (mmho) rise shown.

copper is associated from the livers of rats administered fairly high doses of copper. The resolution of the components obtained by gel filtration which have been studied by other investigators (8,9,21) represents a further step towards complete characterization and hopefully a fuller understanding of the functions of these proteins. In addition to this final purification step we have taken particular care to avoid solvent fractionation and other methods of protein precipitation before the gel filtration step, relying instead on very high speed centrifugation and ultrafiltration.

Although all three proteins isolated are in the same molecular weight range as the metallothioneins (5,6,9,10) the two groups of proteins differ drastically in total amino acid compositions. In fact the large deviation from the approximately 30% cysteine content on the basis of which metallothionein derived its name (1) makes it unlikely that a very significant amount of metallothionein is present in copper-loaded liver. In this respect it is noteworthy that livers from animals administered no trace metals contain very little metallothionein and much less of the copper associated proteins than described here (26).

As Evans (19) has already pointed out, the protein L-6D isolated from the liver of a patient with Wilson's disease by Scheinberg and co-workers (25) seems to be more similar to the proteins appearing on copper loading than a metallothionein. Although further characterization of each of the three purified copper proteins is required to confirm or disprove this similarity, it is tempting to speculate that increased amounts of such proteins may also be synthesized in livers in this pathological state and possibly others (27).

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