Environmental Health Perspectives
Vol. 65, pp. 93-100, 1986

Copper-Metallothioneins in the American Lobster, *Homarus americanus*: Potential Role as Cu(I) Donors to Apohemocyanin

by Marius Brouwer,* Patrick Whaling,* and David W. Engel†

The physiological function of copper(I)-metallothionein is not well understood. The respiratory function of hemocyanin, a copper(I)-containing respiratory protein found in the hemolymph of many invertebrates, has been known a long time. However, the mechanism by which Cu(I) is inserted into the oxygen-binding site of apohemocyanin is completely unknown. This investigation tests the hypothesis that copper(I)-metallothionein may act as a Cu(I) donor to apohemocyanin. To this end, copper-binding proteins and hemocyanin were purified from the digestive gland and hemolymph of the American lobster, *Homarus americanus*. In the presence of β-mercaptoethanol, the copper-binding proteins can be resolved into three components on DEAE-cellulose. The first two have been characterized as metallothioneins, based on their high cysteine content and lack of aromatic amino acid residues. The cysteine content of the third component is half of that of components I and II. In the absence of β-mercaptoethanol the three proteins elute as a single protein complex during ion-exchange chromatography. Components I and II show a strong tendency to polymerize, a process that is accompanied by the loss of protein-bound copper. The purified proteins are not capable of transferring Cu(I) to the active sites of completely copper-free apohemocyanin. They are capable, however, of transferring Cu(I) to active sites of hemocyanin containing reduced amounts of Cu(I), suggesting that the conformational state of hemocyanin is the determining factor in the Cu(I) transfer mechanism.

Introduction

Metallothionein is a low-molecular weight, cysteine- and metal-rich protein. In view of its widespread occurrence, and its conserved molecular structure throughout evolution, it seems likely that this protein serves an important physiological function. However, despite the fact that metallothioneins and the regulation of metallothionein genes have been studied extensively during the past 27 years (*1–3*), there is still uncertainty and controversy about the major biological function(s) of these metal-binding proteins. Much of the earlier work focused on the role of metallothioneins in heavy metal detoxification (*1*). Recently, attention has turned to the role in "normal" zinc and copper metabolism. Brady and Webb (*4*) demonstrated that the levels of (Zn, Cu)-metallothionein changed in the liver, kidneys, and testes of the neonatal and developing rat. Their data are consistent with the hypothesis that high levels of metallothionein are required in tissues which rapidly synthesize metal-containing macromolecules involved in nucleic acid metabolism, protein synthesis, and other metabolic processes. This hypothesis is supported further by the observation that regenerating liver, after partial hepatectomy, requires a high concentration of Zn-metallothionein (*5*). In general, various unrelated physiological stresses, which are accompanied with a change in Zn distribution, increase the synthesis of metallothionein in rat liver (*6*).

Initial studies on the function of metallothionein by Udom and Brady (*7*) and Li et al. (*8*) showed that Zn-metallothionein can transfer Zn to a variety of apoenzymes. In all cases the postulated mechanism of metal transfer involved a direct interaction between metallothionein and apoenzyme. It also has been shown, however, that zinc salts can be used to reactivate apoenzymes which indicates that the thionein complex is not required for zinc transfer. Reconstitution of apo-copper proteins, however, with freshly isolated Cu(I)-metallothionein have not been successful. Only aged Cu-metallothionein, whose copper and sulfhydryl groups had been oxidized, was able to reactivate aposuperoxide dismutase and to bind to apocarbonic anhydrase (*9*). Similarly, freshly prepared Cu-metallothionein from Neurospora could not reactivate apotyrosinase and apohemocyanin (*10*).

Even though the function of Cu-metallothionein has not been demonstrated convincingly, correlative evidence

*Duke University Marine Laboratory, Marine Biomedical Center, Beaufort, NC 28516.
†National Marine Fisheries Service, NOAA, Southeast Fisheries Center, Beaufort Laboratory, Beaufort, NC 28516.

suggests a link between Cu-metallothionein and hemocyanin synthesis in marine crustaceans. Hemocyanin, a Cu(I)-containing protein that serves as an oxygen carrier in crustacea, usually occurs in very high concentrations (up to 100 mg/mL) in the hemolymph. However, both the molt cycle and starvation have been shown to drastically affect the hemocyanin levels in the hemolymph (*12–15*). It is clear, therefore, that marine crustacea have a very active copper metabolism and require an abundant source of Cu(I). Since Cu-metallothionein (*16*) and hemocyanin (*11*) both contain Cu(I) and since hemocyanin can only be reconstituted with Cu(I) (*11*), it is tempting to postulate a physiological connection between the two Cu(I) proteins. In the digestive gland of the American lobster, *Homarus americanus*, large amounts of copper and copper-binding proteins had been demonstrated (*17–19*), and this organ also had been shown to be the site of hemocyanin biosynthesis (*20*). Moreover, Johnston and Barber (*21*) were able to reconstitute apohemocyanin from the spiny lobster, *Panulirus interruptus*, with a crude copper source derived from the digestive gland.

The purpose of this investigation was to purify and characterize the copper-metallothionein obtained from the digestive gland of the American lobster. Additionally, data are presented on the potential role of Cu(I)-metallothionein as a Cu(I) donor to the active site of apohemocyanin.

Materials and Methods

Lobsters were obtained as described by Engel and Brouwer (*17*). Digestive glands were removed from lobsters and used immediately or stored at −60°C. For the preparation of the cytosol, digestive glands were homogenized with a Brinkman Polytron homogenizer in 1 volume (*w/w*) 20 mM Tris, pH 8, and 0.1 mM phenylmethyl sulfonylfluoride at 4°C. The different procedures used in the purification of cytosolic copper-binding proteins are summarized in Figure 1.

Copper-binding proteins were further characterized by gel electrophoresis on 12.5% polyacrylamide gels, using the buffer system as described by Laemli (*23*). Analysis of amino acid compositions, after performic acid oxidation, were carried out by Sequemat (Watertown, MA). The presence of aromatic amino acid residues was determined with a SPEX/Fluorolog 2 fluorescence spectrophotometer.

Tissue copper concentrations were determined by drying 0.5 g of homogenized tissue at 90°C, followed by combustion at 500°C for 18 hr. The ash was dissolved in 10 mL concentrated nitric acid, taken to near dryness, diluted with deionized water, and analyzed by flame aspiration on a Perkin-Elmer 5000 atomic absorption spectrophotometer.

Hemocyanin was prepared by collecting hemolymph and allowing it to clot. The clotted hemolymph was homogenized and centrifuged at 27,000*g* at 4°C for 10 min. The clear supernatant was recentrifuged at 106,000g for 7 hr to pellet the hemocyanin. Pellets were dissolved in 50 mM Tris, 10 mM $CaCl_2$, pH 8, I = 0.13, and used directly, or lyophilized in the presence of sucrose (sucrose/hemocyanin = 2.75 *w/w*). Apohemocyanin was prepared by mixing the protein in 50 mM Tris, pH 8, 10 mM $CaCl_2$, with an equal volume of buffer containing 20 mM KCN. To prepare hemocyanin samples with different amounts of bound copper, the protein was either incu-

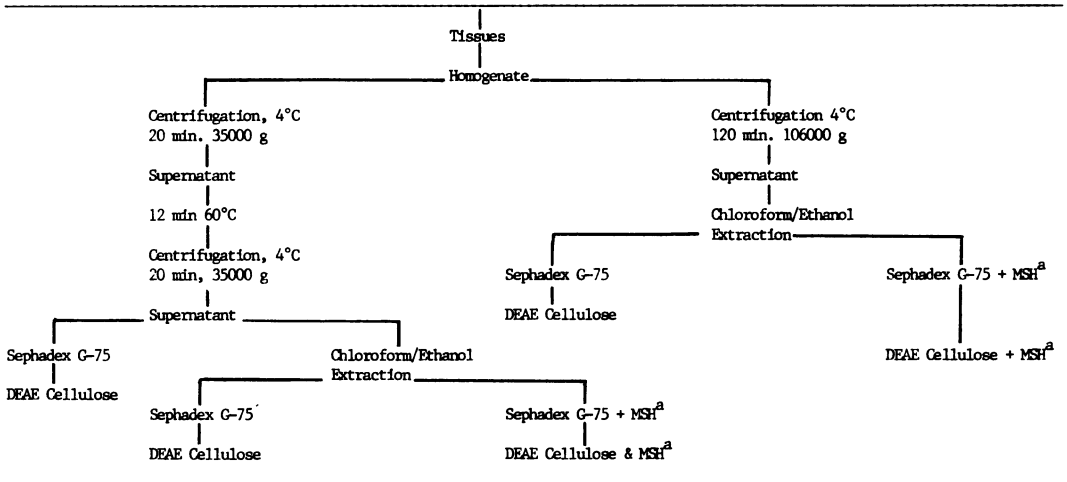

FIGURE 1. Procedures for the purification of copper-metallothioneins from lobster hepatopancreas. Tissue homogenates were centrifuged at 35,000*g* for 20 min. The supernatant was heated at 60°C for 12 min, cooled in ice to 0°C, and centrifuged again at 35,000*g* for 20 min. The supernatant was either applied directly to a column of Sephadex G-75 or subjected to the chloroform–ethanol extraction procedure as described by Winge et al. (*22*). The material obtained after the acetone precipitation was dissolved in 2 mL 20 mM Tris, pH 8, or in 2 mL 20 mM Tris, 2 mM β-mercaptoethanol (MSH), pH 8, and chromatographed on Sephadex G-75. To examine the effect of the 60°C heat step, some cytosols were prepared by centrifugation at 106,000*g* for 2 hr, instead of heating to 60°C. The supernatant was then treated according to Winge's procedure (*22*) and subjected to gel-permeation chromatography with or without β-mercaptoethanol. Copper-containing fractions eluting in the 10,000 M_r region were pooled and applied directly to a DEAE–cellulose De-52 ion-exchange column. All chromatographic procedures were carried out at 4°C using N_2 saturated buffers. Copper proteins obtained after ion-exchange chromatography were concentrated on an Amicon YM-2 membrane, dialyzed against N_2-saturated distilled water or 2 mM β-mercaptoethanol and lyophilized.

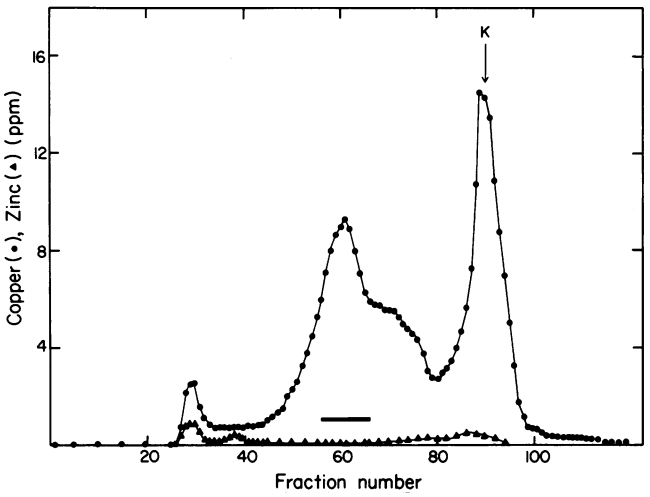

FIGURE 2. Sephadex G-75 elution profile of the cytosolic fraction prepared from frozen lobster digestive gland tissue. Tissues were homogenized, centrifuged for 20 min at 35,000*g*, exposed for 12 min at 60°C, and centrifuged again. No β-mercaptoethanol present. The column (4 × 95 cm) was eluted with 20 mM Tris, pH 8, 4°C, at a flow rate of 80 mL/hr. Fraction volume was 14 mL.

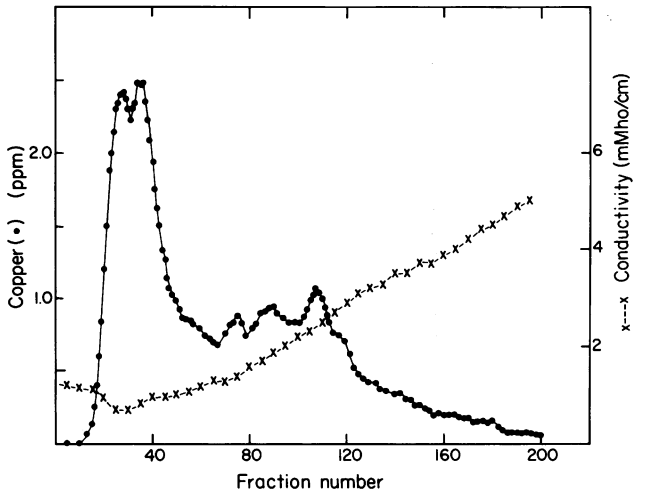

FIGURE 3. DEAE–cellulose elution profile of the pooled fractions obtained after Sephadex G-75 chromatography of lobster cytosol (Fig. 2). A column of 2.5 × 16 cm was developed with a gradient generated from 500 mL 20 mM Tris and 500 mL 300 mM Tris, pH 8, at 80 mL/hr. Fraction volume was 9 mL. No β-mercaptoethanol present.

bated with KCN for 10 min at room temperature, or dialyzed for 30 min against 20 mM KCN, followed by removal of the KCN on Sephadex G-25. Reconstitution of apohemocyanin was performed by mixing the apoprotein with purified copper proteins in 50 mM Tris, pH 8, 10 mM $CaCl_2$, and by following spectrophotometrically the appearance of the copper-oxygen charge transfer band at 335 nm (see Fig. 8 for details). Hemocyanin concentration was calculated from the optical density at 280 nm, using $E^{1\%}_{1cm} = 14.3$ and a value of 75,000 for the molecular weight of a single oxygen-binding site carrying subunit (*24*).

Results

The Sephadex elution profile of a homogenized tissue sample subjected to a 60°C heat step, followed by low-speed centrifugation is shown in Figure 2. Metallothionein represents 36% of the total copper in the chromatogram [also see Engel and Brouwer (*17*), Fig. 5]. Low-molecular weight copper is a predominant copper species in this sample (Table 1). The majority of the copper-binding proteins obtained after Sephadex G-75 chromatography does not bind to the ion-exchange column (Fig. 3). Somewhat similar chromatographic characteristics are obtained when the homogenate, after heat treatment and centrifugation, is subjected to the chloroform–ethanol extraction procedure (Figs. 4 and 5; Table 1). The material obtained after the ion-exchange chromatography does not fluoresce when excited at 280 nm, demonstrating the lack of aromatic amino acid residues in the sample. The amino acid composition of this material (Fig. 5) is shown in Table 2. When 2 mM β-mercaptoethanol is added to the elution buffer, no significant

Table 1. Preparation of copper-binding proteins from the digestive gland of the American lobster, *Homarus americanus*.

	Preparation A, μgCu[a,b]	Preparation B μgCu[a,b]	Preparation C μgCu[a,b]	Preparation D μgCu[a,b]
Homogenate	284	400	369	234
Cytosol	177	86	54	95
Sephadex G-75				
HMW[c]	5	1	2.7	15
MT[d]	57	60	34	30
LMW[e]	96	41	27	30
DEAE–Cellulose				
0.9 mMho/cm	23.4	20	5.4	5.2
1.8 mMho/cm	4.7	–	8.9	11
2.4 mMho/cm	3.9	–	4.8	4.1

[a] Preparation A: frozen tissue, homogenization, low-speed centrifugation, 60°C, low-speed centrifugation (see Figs. 2 and 3); Preparation B: frozen tissue, homogenization, low-speed centrifugation, 60°C, low-speed centrifugation, chloroform–ethanol extraction, acetone precipitation (see Figs. 4 and 5); Preparation C: frozen tissue, homogenization, high-speed centrifugation, chloroform–ethanol extraction, acetone precipitation, 2 mM β-mercaptoethanol in buffers used for chromatography; Preparation D: fresh tissue treated as Preparation C (see Figs. 6 and 7).

[b] Copper concentrations are given as μg Cu per gram wet weight.

[c] HMW: high-molecular weight, void volume fraction.

[d] MT: metallothionein fraction.

[e] LMW: low-molecular weight fraction (anything eluting downstream from metallothionein).

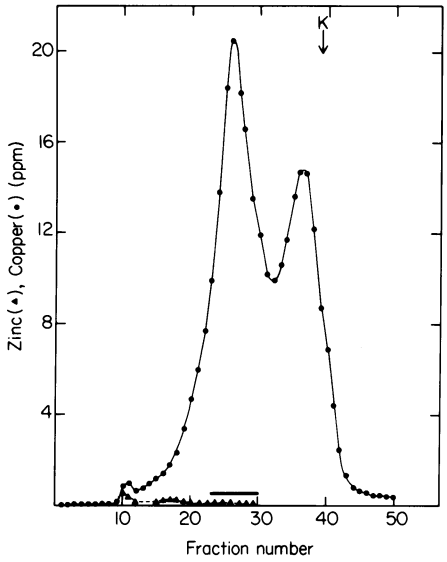

FIGURE 4. Sephadex G-75 elution profile of the cytosolic fraction prepared from frozen lobster digestive gland. Tissues were homogenized, centrifuged for 20 min at 35,000*g*, exposed for 12 min at 60°C, centrifuged again, and then subjected to the chloroform-ethanol extraction procedure. No β-mercaptoethanol present. The column (2.5 × 55 cm) was eluted with 20 mM Tris, pH 8, 4°C, at a flow rate of 35 mL/hr. Fraction volume was 8 mL.

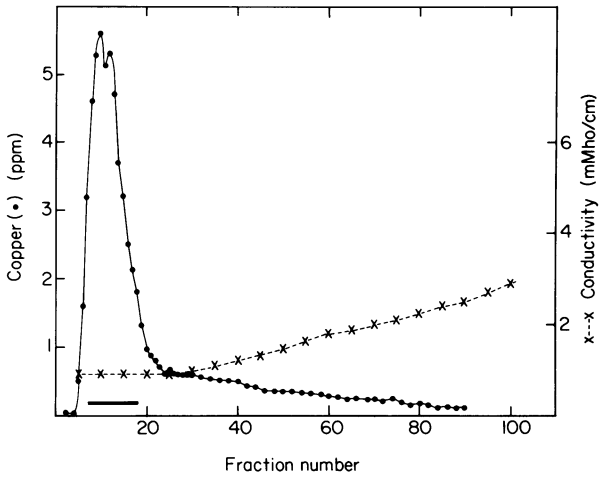

FIGURE 5. DEAE-cellulose elution profile of the pooled fractions obtained after Sephadex G-75 chromatography of lobster cytosol (Fig. 4). Conditions as in Fig. 3.

changes in copper distribution in the Sephadex elution profile are observed. The percentage of the total copper in a Sephadex G-75 chromatogram bound to metallothionein prepared according to Winge's procedure (*22*) is 40% ± 10 (N = 4) with mercaptoethanol, and 54% ± 14 (N = 5) without mercaptoethanol. Also, no significant differences were observed between samples prepared by heat treatment or high-speed centrifugation. The latter samples showed, however, a tendency to contain more of the high molecular weight copper (Fig. 6), which shows that heat treatment denatures high molecular weight copper proteins. When 2 mM mercaptoethanol was present during the ion-exchange chromatography, however, the elution profile of copper proteins was changed dramatically. Instead of the majority of the copper eluting in the breakthrough, three distinct copper-binding proteins were observed (Fig. 7). The ratio of the three proteins, calculated from the copper distribution for three ion-exchange runs, was 24(± 4.6):48.6(± 4.6): 27(± 7.2). None of the proteins showed any fluorescence when excited at 280 nm. Their amino acid composition is shown in Table 2. The relationship between the amino acid composition of these three proteins and that of the single protein obtained in the absence of β-mercaptoethanol will be addressed in the Discussion.

Gel electrophoresis of the three copper-binding proteins, I, II, and III, after concentration, dialysis and

Table 2. Amino acid compositions of *Homarus americanus* copper-binding proteins

	Amino acid content[a,b]				
	I	II	III	IV	V
Cys	23.4	25.5	11.02	19.4	21.1
Asp	6.7	10.7	14.9	10.0	10.9
Thr	7.3	5.6	6.2	7.5	6.2
Ser	7.0	13.1	7.8	8.8	10.2
Glu	9.0	5.3	15.5	8.1	8.9
Pro	6.6	5.1	5.6	6.6	5.6
Gly	12.8	14.7	10.9	13.4	13.2
Ala	7.0	6.6	7.6	6.6	7.0
Val	2.0	1.4	6.6	2.8	2.9
Met	ND[c]	ND	ND	ND	–
Ile	0.8	0.7	2.9	2.0	1.3
Leu	1.5	0.9	3.4	2.2	1.7
Tyr	–	–	–	–	–
Phe	–	–	–	–	–
His	–	–	0.9	–	0.2
Lys	13.0	8.4	4.6	10.9	8.9
Arg	3.0	2.1	2.0	2.0	2.3

[a] The values are given as percentage of the total number of residues.
[b] Fractions: I, Fig. 7, Fractions 10–20; II, Fig. 7, Fractions 47–60; III, Fig. 7, Fractions 70–79; IV, Fig. 5, Fractions 7–18; V, calculated from I, II, and III assuming I:II:III = 0.24:0.49:0.27 (see "Discussion").
[c] Not determined.

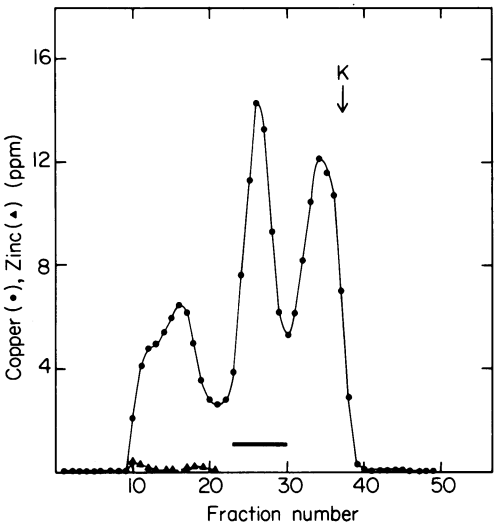

FIGURE 6. Sephadex G-75 elution profile of the cytosolic fraction prepared from fresh lobster digestive gland. Tissues were homogenized, centrifuged for 120 min at 106,000*g*, subjected to the chloroform-ethanol extraction procedure and chromatographed on Sephadex G-75 as described in Fig. 4, in the presence of 2 mM β-mercaptoethanol.

lyophilization did not show clear banding patterns. Protein I moved either as a single band with an R_f value of 0.4 or showed a smear of polymerized material in the upper part of the gel. Protein II showed a protein smear in the upper part of the gel, whereas protein III moved along with the tracking dye front and in addition showed a faint band with an R_f value of 0.7. The aggregated proteins could be depolymerized to some extent by incubation with β-mercaptoethanol or dithiothreitol. Protein I still formed a smear in the middle part of the gel, but protein II showed two closely spaced bands with R_f values of 0.52 and 0.55, respectively.

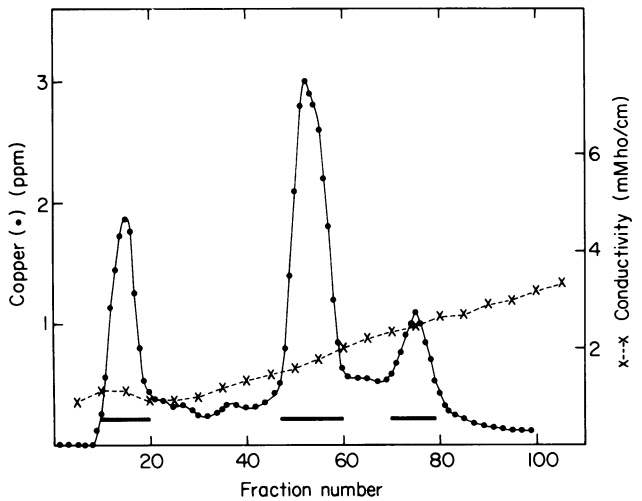

FIGURE 7. DEAE-cellulose elution profile of the pooled fractions obtained after Sephadex G-75 chromatography of lobster cytosol (Fig. 6). Conditions as in Fig. 3, except for the presence of 2 mM β-mercaptoethanol.

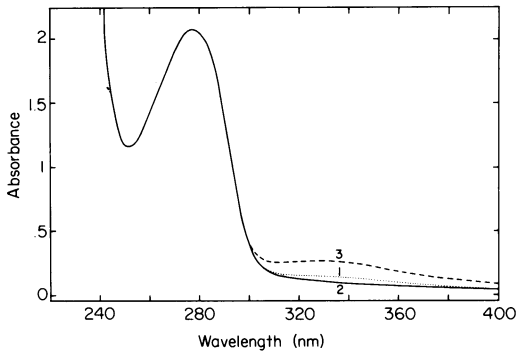

FIGURE 8. Incubation of partial apohemocyanin (17.6 μM), still containing 35% of its original copper, with copper metallothionein (peak I in Fig. 7): (*1*) spectrum in presence of oxygen, immediately after mixing apohemocyanin and copper-metallothionein; (*2*) spectrum in absence of oxygen, after mixing apohemocyanin and copper-metallothionein (after 20 hr of incubation in absence of oxygen, the same spectrum is observed); (*3*) spectrum after 20 hr of incubation in absence of oxygen, followed by 10 min of equilibration with oxygen. Copper-metallothionein concentration expressed as copper was 36 μM.

To study a possible role of the purified copper protein as Cu(I) donors to apohemocyanin, a portion of the copper was removed from the oxygen binding site of hemocyanin. This modified protein, which still contained 35% of its original copper, was incubated with copper-metallothionein (36 μM in copper, Peak I in Fig. 7). The UV absorption spectrum of this mixture in the presence of air is shown as the dotted line (1) in Figure 8. After deoxygenation spectrum 2 was recorded. The decrease at 335 nm upon removal of oxygen indicates that the hemocyanin still contains a small amount of intact, binuclear copper sites. Since the ratio of 280 nm/335nm for fully oxyhemocyanin is 4.5 under the experimental conditions (M. Brouwer, unpublished results), it can be calculated that the fractional saturation of hemocyanin with O_2 is 0.13 (see "Discussion" for details). After 20 hr of incubation of hemocyanin and metallothionein under deoxy conditions, hemocyanin was equilibrated with air and spectrum 3 was recorded. The copper-oxygen change transfer band at 335 nm has increased, and the fractional saturation of hemocyanin with O_2 was 0.40. Therefore, 27% new oxygen binding sites have been created during the incubation period. No such increase is observed when apohemocyanin is incubated under deoxy conditions with $CuSO_4$.

Discussion

Copper-metallothioneins isolated from the livers of mammals are subject to uncontrolled oxidation, leading to disulfide-bridged polymeric species and the loss of copper (*25,26*). Due to the polymerization process, the electrophoretic patterns of these proteins often show diffuse rather than clearly resolved bands (*27,28*). It is imperative, therefore, to carry out the purification of copper-metallothioneins under anaerobic and reducing conditions. Aerobic preparations of rat liver copper metallo-

thionein, for example, contain 10 to 12 titrable sulphhydryl groups, whereas anaerobic preparations contain 18 titrable cysteines (*9*). The results from the current experiments show that the low-molecular weight copper-binding proteins obtained from the digestive gland of the American lobster are similarly sensitive to oxidation. Even preparations purified under nitrogen and in the presence of β-mercaptoethanol have a strong tendency to lose copper during the concentration step on Amicon YM-2 membranes. Also, lyophilization often gives rise to protein preparations that are only partially soluble in buffer and streak during gel electrophoresis.

The necessity of maintaining reducing conditions during the purification procedure is most clearly borne out by the results presented in Figures 3 and 5 and Tables 1 and 2. Without β-mercaptoethanol most of the copper-binding protein(s) elute from the ion-exchangers prior to the gradient, whether conventional or chloroform/ethanol extraction procedures are used (Table 1, Figs. 3 and 5). This was found to be true for both frozen and fresh tissues. As mentioned previously, in the presence of β-mercaptoethanol the copper-binding proteins could be resolved into three components, occurring in a ratio of 24 ± 5, 49 ± 5, and 27 ± 7 (N = 3) (Fig. 7). The amino acid compositions of these three proteins and the protein purified in the absence of β-mercaptoethanol are given in Table 2. The most striking feature of Table 2 is the fact that the amino acid composition of the "oxidized" protein isolated without mercaptoethanol is very close to the weighted average amino acid composition of the three proteins resolved under reducing conditions. This observation strongly suggests that the copper-binding protein purified in the absence of β-mercaptoethanol is an aggregate of three different proteins, which is further substantiated by gel electrophoresis. The "oxidized" protein shows a smear in the upper part of the gel, whereas component III (eluting at 2.4 mMho/cm) of the "reduced" protein moves with the tracking front dye. When the "oxidized" protein is reduced with 14 mM dithiothreitol for 1 hr, a large diffuse band is observed in the middle part of the gel, together with the fast moving component III.

The purified lobster low molecular weight copper-binding proteins have many of the attributes of the mammalian metallothioneins. For example, none of the purified lobster proteins showed fluorescence when excited at 280 nm, indicating the absence of aromatic amino acid residues, which is characteristic of metallothioneins. Also, components I and II (eluting at 0.9 and 1.8 mMho/cm, respectively) show high cysteine contents, comparable to those of other metallothioneins. Component III, however, contains less cysteine. These results are strikingly similar to those obtained for human fetal liver copper-metallothionein (*28*), where the copper protein eluting last from the ion-exchanger had a lower cysteine content than the proteins eluting at lower conductivities. In general, the results obtained from lobster digestive gland are very similar to those published for mammalian systems. Rat, human fetal, and pig liver, for example, all contain a copper-metallothionein that does not bind to DEAE–cellulose and two or three copper-metallothioneins that can be resolved in the gradient (*22,27,28*).

Freezing of the tissues did not affect the copper-metallothionein purification (Table 1). Freezing, however, seems to affect the pool of copper, eluting at lower molecular weight than metallothionein. Most of the frozen samples did show two low molecular weight copper pools (Fig. 2), using either conventional or chloroform/ethanol extraction procedures. The 96 μg Cu in the low-molecular weight pool (Table 2) actually consists of 64 μg Cu eluting close to the salt peak and 32 μg Cu eluting in between metallothionein and salt peak (Fig. 2). This distribution of copper over two low molecular weight ranges was never observed in fresh tissues.

The physiological function of metallothionein has not been defined, but some hypotheses concerning its function have been proposed. It has been demonstrated, for example, that zinc-metallothionein can reactivate apoenzymes that require zinc for their biological activity (*7,8*). In contrast, freshly anaerobically prepared copper-metallothioneins, whose coppers are in the Cu(I) oxidation state, are not able to reactivate aposuperoxide dismutase or apotyrosinase, proteins that require copper for their enzymatic activity (*9,10*). These enzymes, however, can be reconstituted with Cu(II) ions (*29,30*). Other copper proteins, such as stellacyanin and azurin, can be reconstituted in the same way (*31–34*). Ceruloplasmin and hemocyanin, on the other hand, can only be reconstituted with Cu(I) (*11*). The process of copper insertion into these proteins *in vivo* remains unclear. Holtzman and Gaumnitz (*35*) demonstrated that, when ^{64}Cu was administered to copper-deficient rats, none of it was bound to the circulating apoceruloplasmin. Instead, copper was inserted in the protein before it was released from the liver in the circulation. Keyhani and Keyhani (*36*) studied the role of copper in the biosynthesis and assembly of active cytochrome c oxidase in *Candida utilis* yeast cells. They found that the apoprotein of cytochrome c oxidase is synthesized and inserted in the membrane of copper-deficient cells. When copper was added to the growth medium the metal became incorporated in the protein. However, when copper was added and the cytoplasmic protein synthesis was inhibited at the same time, copper was not inserted in cytochrome c oxidase, indicating that a cytoplasmic protein is required for copper insertion.

In view of the fact that hemocyanin biosynthesis in lobsters occurs in the digestive gland and requires a source of Cu(I), we have investigated the possible role of copper-metallothionein, isolated from the same organ, as Cu(I) donor to apohemocyanin. The results obtained so far seem promising, but rather complex. Most of the reconstitution experiments have been carried out with the copper-metallothionein eluting at 0.9 mMho/cm (Fig. 7). In this paper we will limit ourselves to discuss the results obtained with this protein, but would like to mention that preliminary experiments show that the other two copper proteins behave approximately in the same way as lobster copper-metallothionein I.

When apohemocyanin, containing less than 2% of its original copper, was incubated with copper-metallothionein, in air or *in vacuo*, no increase in intact oxygen-binding sites was observed. The same phenomenon was observed when partial apohemocyanin was incubated with copper metallothionein in air. However, when partial apohemocyanin, which still contained 35% of its copper, was incubated with copper-metallothionein, for 15 to 20 hr, in the absence of oxygen, the number of intact oxygen-binding sites increased (Fig. 8). Appropriate controls, using buffer or Cu(II) containing buffer, did not show any reconstitution. The number of intact oxygen-binding sites prior to, and after reconstitution, can be calculated from the absorbance at 335 nm, which is due to the copper-oxygen complex. The spectrum of the partial apohemocyanin is recorded in the absence of oxygen and then after equilibration with oxygen. This gives the value of ΔOD_{335}

$$(OD_{335,oxy}) - (OD_{335,deoxy}) = \Delta OD_{335} \quad (1)$$

for the partial apohemocyanin. The value of $OD_{335,fully\ oxy}$ is calculated from the experimentally determined relationship for the intact protein: $OD_{280}/OD_{335} = 4.5$. This gives us the value of $\Delta OD_{335,max}$

$$(OD_{335,fully\ oxy}) - (OD_{335,deoxy}) = \Delta OD_{335,max} \quad (2)$$

of the native protein. The fraction of intact binding sites is then equal to $\Delta OD_{335}/\Delta OD_{335,max}$. The results presented in Figure 8 show that the fraction of intact oxygen-binding sites has increased from 0.13 to 0.40, after incubation with copper-metallothionein.

The inability to reconstitute completely copper-free apohemocyanin suggests that removal of all copper results in a conformational change of protein structure, making the active sites inaccessible to copper(I). Such conformational changes in hemocyanin have been postulated by Salvato and Zatta, who demonstrated that copper removal from hemocyanin occurs in discrete steps. First, the holoprotein, containing binuclear-copper sites, is converted to the half-apoprotein, which contains mononuclear-copper sites. The protein then undergoes a conformational change followed by the release of the second copper (*37*). Since "partial" apohemocyanin, used in our experiments, contains mononuclear-copper or half-apo sites, the possibility exists that only these sites are amenable to reconstitution. The fraction of mononuclear-copper sites in partial apohemocyanin can be calculated from the amount of copper still present and the fraction of binuclear-copper sites. No correlation between mononuclear-copper sites and reconstitution was found. For example, the fraction of mononuclear-copper sites in the experiment shown in Figure 8 was calculated to be 0.44, whereas the increase in intact binding sites amounts to 0.27. The percentage reconstitution could not be increased by increasing the copper-metallothionein concentration, strongly suggesting that the amount of mononuclear-copper sites is not the determining factor in the reconstitution process.

As indicated previously, investigations have been conducted where reconstitution of copper protein was successfully done with Cu(II). The general picture that emerges from these studies is that the reconstitution occurs by a two-step process. In the first step, the copper is bound rapidly to the protein at a site that is spectroscopically different from the native one. The second step is a slow, rate-limiting conformational change of the protein after which all the spectroscopic properties of the native protein are regained (*29–34*). Preliminary studies carried out in our laboratory also indicate that protein conformational changes are important in the reconstitution process. We have found, for example, that it is more difficult to reconstitute freshly prepared partial apohemocyanin than one- or two-day-old preparations. This change in reconstitution potential is accompanied by a conformational change of the protein as judged from a distinct decrease of the tryptophan fluorescence intensity. Such observations seem to suggest that sudden removal of copper out of the active site of hemocyanin alters the structure of these sites, making them temporarily inaccessible to Cu(I). With time the sites slowly relax to a conformation that is capable of accepting the metal.

The results reported here are consistent with some of the data published by Beltramini and Lerch (*10*), who demonstrated that a freshly isolated copper-metallothionein from Neurospora was incompetent in transferring the metal ions to apohemocyanin from *Cancer magister* in the presence of oxygen. However, Beltramini and Lerch were able to reconstitute apohemocyanin with aged, oxidized copper-metallothionein, after reduction of Cu(II) to Cu(I) with dithionite. Addition of dithionite to our incubation mixtures of apohemocyanin and copper-metallothionein did not result in enhancement of copper transfer. Moreover, mixtures of Cu(II) and dithionite did not result in reconstitution of lobster hemocyanin.

In summary, we may say that copper-metallothioneins isolated from the digestive gland of the lobster clearly have the potential to transfer copper to the active site of apohemocyanin prepared from the same organism. Unfortunately, the transfer process seems to be rather slow and limited. Studies to better understand and hence speed up the rate limiting steps in the copper transfer from copper-metallothionein to apohemocyanin are under way.

The authors thank Dr. F. P. Thurberg of the National Marine Fisheries Service, Milford Laboratory, Milford, CT, for furnishing the lobsters used in this investigation. The research data presented in this paper was funded by NOAA's National Ocean Service, Ocean Assessment Division, through a cooperative research program with the Southeast Fisheries Center's Beaufort Laboratory and the Duke University Marine Laboratory/Marine Biomedical Center.

REFERENCES

1. Kägi, J. H. R., and Nordberg, M. (Eds.). Metallothionein. Birkhauser Verlag, Basel, 1979.
2. Foulkes, E. C. (Ed.). The Biological Roles of Metallothionein. Elsevier/North-Holland, 1982.
3. Brady, F. O. The physiological function of metallothionein. Trends Biochem. Sci. 7: 143–145 (1982).

4. Brady, F. O., and Webb, M. Metabolism of zinc and copper in the neonate. J. Biol. Chem. 256: 3931–3935 (1981).
5. Ohtake, H., Hasegawa, K., and Koga, M. Zinc-binding protein in the livers of neonatal, normal and partially hepatectomized rats. Biochem. J. 174: 999–1005 (1978).
6. Oh, S. H., Deagen, J. T., Whanger, P. D., and Weswig, P. H. Biological function of metallothionein. V. Its induction in rats by various stresses. Am. J. Physiol. 234: E282–E285 (1978).
7. Udom, A. O., and Brady, F. O. Reactivation *in vitro* of zinc-requiring apo-enzymes by rat liver zinc-thionein. Biochem. J. 187: 329–335 (1980).
8. Li, T. Y., Kraker, A. J., Shaw, C. F., and Petering, D. H. Ligand substitution reactions of metallothioneins with EDTA and apocarbonic anhydrase. Proc. Natl. Acad. Sci. (U.S.) 77: 6334–6338 (1980).
9. Geller, B. L., and Winge, D. R. Metal binding sites of rat liver Cu-thionein. Arch. Biochem. Biophys. 213:109–117 (1982).
10. Beltramini, M., and Lerch, K. Copper transfer between Neurospora copper metallothionein and type 3 copper apoproteins. FEBS Letters, 142: 219–222 (1982).
11. Lontie, R., and Witters, R. Hemocyanin. In: Inorganic Biochemistry, Vol. 1 (G. L. Eichhorn, Ed.), Elsevier Scientific, Amsterdam, New York, 1973, pp. 344–358.
12. Zuckerkandl, E. Hemocyanine et cuivre chez un crustace decapode, dans leurs rapports avec le cycle d'intermue. Ann. Inst. Oceanogr., Monaco T-38: 1–22 (1960).
13. Djangmah, J. S. The effects of feeding and starvation on copper in the blood and hepatopancreas and on blood proteins of *Crangon vulgaris*. Comp. Biochem. Physiol. 32: 709–731 (1970).
14. Djangmah, J. S., and Grove, D. J. Blood and hepatopancreas copper in *Crangon vulgaris*. Comp. Biochem. Physiol. 32: 733–745 (1970).
15. Hagerman, L. Haemocyanin concentration of juvenile lobsters (*Homarus gammarus*) in relation to moulting cycle and feeding conditions. Mar. Biol. 77: 11–17 (1983).
16. Nielson, K. B., and Winge, D. R. Preferential binding of copper to the βdomain of metallothionein. J. Biol. Chem. 259: 4941–4946 (1984).
17. Engel, D. W., and Brouwer, M. Cadmium and copper metallothioneins in the American lobster, *Homarus americanus*. Environ. Health Perspect. 65: 87–92 (1986).
18. Engel, D. W., and Brouwer, M. Trace metal-binding proteins in marine molluscs and crustaceans. Mar. Environ. Res. 13: 177–194 (1984).
19. Engel, D. W., Brouwer, M., and Thurberg, F. P. Comparison of metal metabolism and metal-binding proteins in the blue crab and the American lobster. In: Marine Pollution and Physiology: Recent Advances (F. J. Vernberg, A. Calabrese, F. P. Thurberg, and W. B. Vernberg, Eds.). University of South Carolina Press, Columbia, SC, 1985, pp. 229–245.
20. Senkbeil, E. G., and Wriston, J. C., Jr. Hemocyanin synthesis in the American lobster, *Homarus americanus*. Comp. Biochem. Physiol. 68B: 163–171 (1981).
21. Johnston, W., and Barber, A. A. Reconstitution of functional hemocyanin from apohemocyanin: the hepatopancreas as copper donor. Comp. Biochem. Physiol. 28: 1259–1273 (1969).
22. Winge, D. R., Geller, B. L., and Garvey, J. Isolation of copper thionein from rat liver. Arch. Biochem. Biophys. 208: 160–166 (1981).
23. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685 (1970).
24. Nickerson, K. W., and Van Holde, K. E. A comparison of molluscan and arthropod hemocyanin I. Circular dichroism and absorption spectra. Comp. Biochem. Physiol. B39: 855–872 (1971).
25. Hartman, H. J., and Weser, U. Copper-thionein from fetal bovine liver. Biochim. Biophys. Acta 491: 211–222 (1977).
26. Minkel, D. T., Poulsen, K., Wielgus, S., Shaw, C. F., and Petering, D. H. On the sensitivity of metallothioneins to oxidation during isolation. Biochem. J. 191: 475–485 (1980).
27. Bremner, I., and Young, B. W. Isolation of (copper, zinc)-thioneins from pig liver. Biochem. J. 155: 631–635 (1976).
28. Ryden, L., and Deutsch, H. F. Preparation and properties of the major copper-binding component in human fetal liver. J. Biol. Chem. 253: 519–524 (1978).
29. Rigo, A., Viglino, P., Brunori, M., Cocco, D., Calabrese, L., and Rotilio, G. The binding of copper ions to copper-free bovine superoxide dismutase. Biochem. J. 169: 277–280 (1978).
30. Kertesz, O., Rotilio, G., Brunori, M., Zito, R., and Antonini, E. Kinetics of reconstitution of polyphenoloxidase from apoenzyme and copper. Biochem. Biophys. Res. Commun. 49: 1208–1215 (1972).
31. Morpurgo, L., Finazzi-Agro, A., Rotilio, R., and Mondovi, B. Studies on the metal sites of copper proteins. IV. Stellacyanin: preparation of apoprotein and involvement of sulfhydryl and tryptophan in the copper chromophore. Biochim. Biophys. Acta 271: 292–299 (1972).
32. Morpurgo, L., Rotilio, R., Finazzi-Agro, A., and Mondovi, B. The role of copper binding in the conformation of stellacyanin. Arch. Biochem. Biophys. 161: 291–296 (1974).
33. Marks, R. H. L., and Miller, R. D. A kinetic study of the reconstitution of azurin from Cu(II) and the apoprotein. Arch. Biochem. Biophys. 195: 103–111 (1979).
34. Blaszak, J. A., McMillin, D. R., Thornton, A. T., and Tennent, D. L. Kinetics of copper(II) uptake by apoazurin in complexing media. J. Biol. Chem. 258: 9886–9892 (1983).
35. Holtzman, N. A., and Gaumnitz, B. M. Studies on the rate of release and turnover of cerulophasmin and apoceruloplasmin in rat plasma. J. Biol. Chem. 245: 2354–2358 (1970).
36. Keyhani, E., and Keyhani, J. Cytochrome c oxidase biosynthesis and assembly in *Candida utilis* yeast cells: function of copper in the assembly of active cytochrome c oxidase. Arch. Biochem. Biophys. 167: 596–602 (1975).
37. Salvato, B., and Zatta, P. Kinetics of reaction between hemocyanin and CN^- and of reconstitution of hemocyanin with $K_3Cu(CN)_4$. In: Structure and Function of Haemocyanin (J. V. Bannister, Ed.), Springer-Verlag, Berlin, 1977, pp. 243–252.