Glutathione-Mediated Transfer of Copper(I) into American Lobster Apohemocyanin†

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ABSTRACT: Copper in the cytosol of the hepatopancreas of the American lobster, *Homarus americanus*, occurs as copper–metallothionein [Cu(I)–MT] and as a copper–glutathione complex [Cu(I)–GSH]. The latter can act in vitro as the source of Cu(I) in the reconstitution of lobster apohemocyanin, whereas Cu(I)–MT cannot. Here we report on the mechanism of the GSH-mediated reconstitution. Binding of Cu(I) to apohemocyanin was measured by its effect on the protein's fluorescence, by ultrafiltration experiments and size-exclusion HPLC. Reconstitution of CO and O_2 binding was studied using the [Cu(I)···Cu(I)–CO] fluorescence of hemocyanin and its Cu–O_2–Cu charge-transfer band as spectral probes. The hemocyanin oligomer has 1 (1.02 ± 0.09) high-affinity (apparent K_{diss} = 1.67 ± 0.40 μM) external binding site for ionic Cu(I) per subunit. Binding of Cu(I) to this site is fast and reversible and is followed by a slow, irreversible incorporation of copper into the protein matrix. Movement of the first copper through the matrix to the active site is the rate-limiting step in the reconstitution process. Mononuclear copper sites, once formed, are rapidly converted into biologically active, binuclear copper sites. In accordance with this reaction sequence, the restoration of CO/O_2 binding by hemocyanin is a first-order reaction with a half-time of 100 ± 5 min at pH 6.0. Reconstitution is extremely pH-dependent and proceeds best at those pH values where the architecture of the copper pocket of hemocyanin is open as judged from its extremely low affinity for oxygen and its very fast oxygen dissociation rate. Our studies suggest that GSH may be involved in mobilization and delivery of Cu(I) for the biosynthesis of copper proteins.

Copper is essential for the biological function of a large number of proteins, which function in electron transport, in oxygen transport, in oxygen activation/substrate oxidation, and in oxygen dismutation (Spiro, 1981). The redox properties that underlie the biological function of copper, when incorporated in a protein matrix, are also responsible for the toxic properties it exhibits when free in solution. For example, copper catalyzes the production of highly toxic hydroxyl radicals from physiologically generated reduced oxygen species, such as superoxide anion and hydrogen peroxide, through a Fenton-like reaction (Samuni et al., 1981). The copper-catalyzed formation of hydroxyl radicals has been implicated in oxidative damage to DNA, proteins, and membranes (Aruoma et al., 1991; Floyd, 1990). It is therefore necessary for organisms to limit the concentration of free copper to a minimum, which raises the question of how copper is transported within an organism and how the metal is made available for incorporation into apometalloproteins.

In mammals, albumin and ceruloplasmin play important roles in the interorgan transport of copper. Albumin's main function seems to be to transport copper from the gut to the liver. Copper is released from the liver as a component of ceruloplasmin and, in time, taken up by extrahepatic organs [see Harris (1991) for a review]. Transmembrane transport of copper involves carrier proteins, which have not yet been purified and characterized (McArdle et al., 1988; Darwish et al., 1983). Whether membrane carriers recognize free ionic copper or copper–amino acid complexes is still unclear. Histidine facilitates copper uptake by some cells, but the amino acid is not cotransported with copper (Darwish et al., 1984). Ceruloplasmin-mediated uptake of copper outside the liver requires a specific interaction between the protein and the membrane, and high-affinity sites for ceruloplasmin have been reported in a number of tissues and cells (Saenko & Yaropolov, 1990; Orena et al., 1986). The copper atoms in ceruloplasmin are reduced prior to release, and Cu(I) is the form of copper taken up by the cells (Dameron & Harris, 1987; Percival & Harris, 1989).

Less is known about intracellular transport of copper than any other phase of its metabolism. Three inherited diseases of copper metabolism are known, suggesting that at least one and possibly three different proteins play an important role in copper metabolism at the cellular level (Palida & Ettinger, 1991). Proteins with apparent molecular masses of approximately 38 and 88 kDa have been shown to bind copper when it first enters hepatocytes (Palida et al., 1990). To date, the low molecular weight metallothioneins (MTs)[1] have received the most attention for possible roles in copper metabolism. They are implicated in metal detoxification and in the delivery of copper for the biosynthesis of metalloproteins (Huber & Lerch, 1987). However, intact, nonoxidized Cu(I)MT cannot directly reactivate copper-dependent apoenzymes and proteins (Geller & Winge, 1982; Beltramini and Lerch, 1982).

The intracellular molecular events involved in the incorporation of copper into the active site of apometalloproteins are under investigation. Kinetic studies on the in vivo incorporation of copper into ceruloplasmin indicate that incorporation occurs early in the course of biosynthesis (Sato & Gitlin, 1991). Studies on the biosynthesis of the copper proteins tyrosinase and laccase have shown that copper ions must be mobilized from CuMT before incorporation into the active site of the apoenzymes. The mechanism of mobilization is unknown (Huber & Lerch, 1987). Recent studies suggest that

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[1] Abbreviations: MT, metallothionein; GSH, glutathione; Tris, 2-amino-2-(hydroxymethyl)-1,3-proanediol; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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glutathione plays a role in delivery of copper to apometallothionein (Freedman et al., 1989) and other copper-dependent proteins (Steinkuhler et al., 1991).

Marine crustacea display an enormously active copper metabolism that is correlated with the turnover of hemocyanin. This protein functions in oxygen transport, and has two Cu(I) atoms in its active site, each of which is ligated to three histidine residues (Gaykema et al., 1986). Dioxygen binds as a μ-peroxo bridge to both coppers (Himmelwright et al., 1980). When crustaceans molt, they may break down as much as two-thirds of their hemocyanin. The copper released in that process must be sequestered to protect the organism from oxidative damage. When hemocyanin is resynthesized, there is a metabolic requirement for this metal. We have demonstrated that hemocyanin synthesis in the blue crab, *Callinectes sapidus*, occurs in the hepatopancreas (Rainer & Brouwer, 1991). We have also shown that the changes in CuMT that occur in the hepatopancreas during the molt cycle of the blue crab can be correlated with the turnover of hemocyanin (Engel & Brouwer, 1987, 1991). These phenomena provide us with a unique opportunity for the study of the dynamics of copper metabolism.

The CuMTs from the blue crab and the American lobster can be separated into three isoforms (Schlenk & Brouwer, 1991; Brouwer & Brouwer-Hoexum, 1991; Brouwer et al., 1992a,b). We found that these proteins do not function as a direct source of Cu(I) in the restoration of the oxygen binding capacity of copper-free hemocyanin in vitro. A copper complex, rich in cysteine and glycine, and possibly derived from glutathione, can reactivate apohemocyanin (Brouwer et al., 1989; Brouwer & Brouwer-Hoexum, 1991). Recently we have demonstrated that the CuMT isoforms from the lobster interact with the tripeptide glutathione (GSH). CuMTIII forms a stable 1:1 complex with GSH, whereas CuMTI/II forms a transient complex, from which copper is released as a copper–glutathione complex. Spectrophotometric titrations of GSH with Cu(I) have shown that the GSH/Cu(I) ratio in the complex is 2 (Brouwer & Brouwer-Hoexum, 1991; Brouwer et al., 1992a). Our studies suggest that a specific CuMT isoform is the source of Cu(I) for the biosynthesis of hemocyanin and that GSH is involved in mobilization and delivery of Cu(I). In the following, we document the role of Cu(I)–glutathione in the reconstitution of copper-free hemocyanin.

Materials and Methods

Preparation of Whole Organ Cytosol and Measurement of Subcellular Partitioning of Copper and Glutathione by Size-Exclusion Chromatography. Three grams of hepatopancreas tissue was homogenized in 3 mL of ice-cold, nitrogen-saturated 10 mM Hepes/50 mM NaCl containing 0.1 mM PMSF, pH 7.6, with a Brinkmann Polytron homogenizer. The homogenate was centrifuged for 60 min at 165000*g*. Acetone (−20 °C) was added to the supernatant (final concentration 40%), followed by centrifugation for 10 min at 15000*g*. The supernatant was made 67% in acetone and centrifuged again for 10 min at 15000*g*. The final pellet was dissolved in 3.5 mL of Hepes/50 mM NaCl, pH 7.6, and chromatographed on Sephadex G-75 (4.2 × 50 cm) in nitrogen-saturated buffer at a flow rate of 35 mL/h. Fractions of 3.5 mL were collected and analyzed for copper by atomic absorption spectroscopy, and for GSH using the enzymatic recycling assay as described by Anderson (1985) and modified by Brouwer and Brouwer-Hoexum (1991).

Reconstitution of Copper-Free Hemocyanin with Cu(I)–Diglutathione. Lobster hemocyanin and the copper-free protein were prepared as described before (Brouwer et al., 1986; Brouwer & Brouwer-Hoexum, 1991). Apohemocyanin oligomers were dialyzed overnight, at 4 °C, vs an appropriate buffer, containing 5 mM $CaCl_2$ to maintain the integrity of the multisubunit hemocyanin aggregate (see figure legends for detail). Apohemocyanin subunits were prepared by dialysis vs 10 mM Tris, pH 8.5, and 1 mM EDTA. In a typical reconstitution experiment, a solution of 10 μM apohemocyanin was incubated in a CO atmosphere with 40 μM Cu(I) and 200 μM glutathione. Cu(I) was added from a 10 mM stock solution of Cu(I), prepared by dissolving $Cu^{I}Cl$ in degassed 0.1 N HCl + 4% NaCl. The concentration of Cu(I) was measured spectrophotometrically at 483 nm as a Cu(I)–bathocuproine disulfonate complex using a molar absorptivity of 12250 (Rifkind et al., 1976). Incorporation of Cu(I) into the active site was determined by its effect on hemocyanin's tryptophan fluorescence (Engel & Brouwer, 1987). Restoration of hemocyanin's CO binding capacity was determined by measurement of the Cu(I)–CO fluorescence emission signal at 580 nm, generated by excitation at 280 nm (Brouwer et al., 1989). Emission spectra from 300 to 700 nm were recorded using a SPEX Fluorolog fluorescence spectrophotometer in the ratio mode. Corrections for inner-filter effects were made according to $Fl_c = Fl_{obs}$ antilog $[(A_{ex} + A_{em})/2]$ (Lakowicz, 1983), where Fl_c and Fl_{obs} are the corrected and observed fluorescence signal, respectively, and A_{ex} and A_{em} are the absorbance at the excitation and emission wavelength, respectively. The fractional saturation of the protein with CO was calculated from $Y_{CO} = (Fl_t - Fl_0)/(Fl_{max} - Fl_0)$, where Fl_{max}, Fl_0, and Fl_t are the values of the fluorescence emission of the fully reconstituted protein, the fluorescence at time zero, and the fluorescence at time t, respectively. The concentration of functionally active oxygen binding sites was determined from the intensity of the copper–oxygen charge-transfer band at 334 nm (A), after addition of oxygen to the degassed incubation mxiture: $Y_{oxygen} = (A_t - A_0)/(A_{max} - A_0)$.

Size-Exclusion HPLC. A solution of 10 μM apohemocyanin was incubated with 40 μM Cu(I) and 200 μM glutathione as described above. Samples of 200 μL were applied to a Spherogel TSK SW3000 column (0.75 × 30 cm), which was eluted with 10 mM Bis-Tris, 5 mM $CaCl_2$, and 100 mM NaCl, pH 6.0, at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected and analyzed for hemocyanin, copper, and glutathione. Hemocyanin concentrations were calculated from the optical density at 280 nm (Brouwer et al., 1986). Copper and glutathione were measured as described above. The same procedure was carried out with holohemocyanin.

Ultrafiltration Experiments. A solution of 5 μM apohemocyanin (or holohemocyanin) in Bis-Tris, pH 6.0, 5 mM $CaCl_2$, and 0.5 M NaCl was concentrated to 10 μM on an Amicon PM-30 membrane in the presence of different amounts of Cu(I)–diglutathione (GSH/copper ratios = 5 and 20). Filtrates were collected 1 min after apohemocyanin was mixed with Cu(I) and glutathione. Retentate and filtrate were analyzed for copper and glutathione, and the hemocyanin concentration in the retentate was determined spectrophotometrically.

Sedimentation Analysis of Lobster Hemocyanin at pH 6.0, 7.5, and 8.5. Sedimentation velocity experiments were carried out with a Beckman Spinco Model E analytical ultracentrifuge, using the photoelectric scanner at 280 nm.

Oxygen Equilibrium and Oxygen Dissociation Experiments. Tonometric oxygen equilibrium experiments were carried out as described by Riggs and Wolbach (1956). The rates of dithionite-induced oxygen dissociation were measured using a Gibson–Durrum stopped-flow apparatus. The instrument

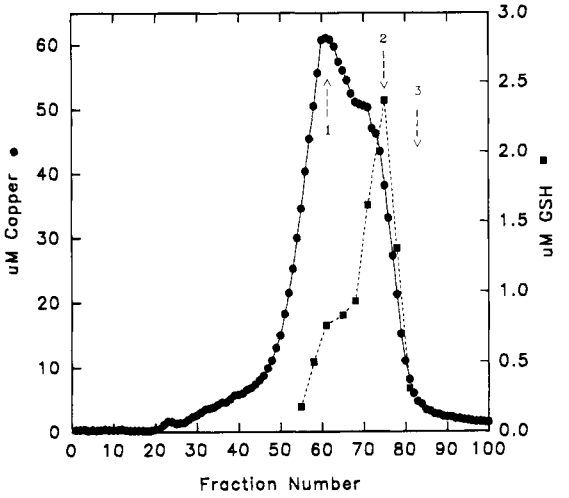

FIGURE 1: Subcellular distribution of glutathione and copper in a cytosolic fraction prepared from acetone-fractionated hepatopancreas tissue from lobster as measured by gel filtration on Sephadex G-75. The V_e/V_0 values for CuMT, Cu(I)–diglutathione, and free GSH were (1) 2.60, (2) 3.26, and (3) 3.48, respectively. Copper (●); glutathione (■).

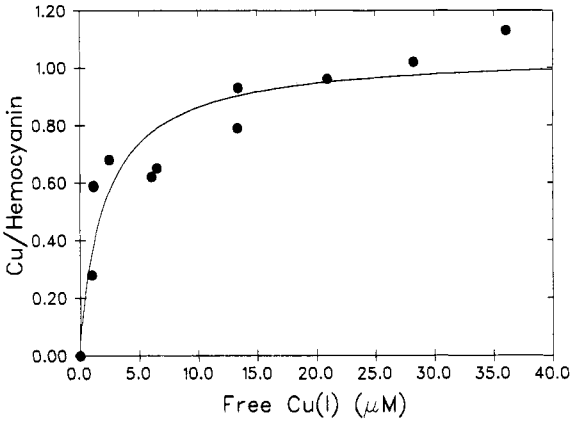

FIGURE 2: Binding of Cu(I) to apohemocyanin as evaluated by ultrafiltration experiments in 10 mM Bis-Tris, pH 6.0, 5 mM $CaCl_2$, and 0.5 M NaCl. A 5-fold molar excess of glutathione over Cu(I) was used in this experiment. The line was calculated with K_{diss} = 1.67 ± 0.40 μM, determined from the data by nonlinear least-squares minimization, with the asumption of one binding site for Cu(I). Scatchard analysis of the data showed 1.02 ± 0.09 binding sites for copper per hemocyanin subunit, with an apparent K_{diss} = 2.14 ± 0.59 μM.

was interfaced to a Tecmar Labmaster data acquisition board in an IBM-AT computer. Data acquisition and analysis were carried out under control of ASYST (ASYST, Software Technologies, Inc.).

Results

Subcellular Distribution of Copper and Glutathione. Gel filtration of an acetone-fractionated tissue homogenate of the hepatopancreas shows that glutathione coelutes with CuMT (V_e/V_0 = 2.6) and with low molecular weight copper. The latter complex elutes in exactly the same position as Cu(I)–diglutathione (V_e/V_0 = 3.26; the V_e/V_0 of free GSH is 3.48, Figure 1). The stoichiometry of glutathione and copper in the low molecular weight fractions is much less than 2, suggesting the presence of copper ligands other than GSH. This observation will be further addressed under Discussion.

Reconstitution of Apohemocyanin with Cu(I)–Diglutathione. To determine optimum conditions for reconstitution of apohemocyanin with Cu(I)–diglutathione, 10 μM apohemocyanin was incubated with 40 μM Cu(I)/200 μM GSH under nitrogen at pH 6 and 6.5 (10 mM Bis-Tris), pH 7 and 7.5 (10 mM Hepes), and pH 8 and 8.5 (10 mM Tris) with and without 0.5 M NaCl. All buffers contained 5 mM $CaCl_2$ to prevent dissociation of the hemocyanin oligomers. After 24 h, the pH of the solutions was raised to pH 8.5, to increase hemocyanin's oxygen affinity, and the samples were equilibrated with pure oxygen. Complete reconstitution of oxygen binding was observed only at pH 6. Subsequent experiments showed that 0.5 M NaCl increased the rate of reconstitution of active sites at pH 6.0 about 3-fold over the rate in the absence of NaCl. Consequently, the reconstitution process was studied in most detail at pH 6.0 in the presence of 0.5 M NaCl.

Binding of Cu(I) to Apohemocyanin. The first step in the Cu(I)–GSH-mediated activation of apohemocyanin involves the binding of Cu(I) [or Cu(I)–diglutathione] to the surface of the protein molecule, followed by internalization of the metal. This process was evaluated by fluorescence spectroscopy, size-exclusion HPLC, and ultrafiltration experiments. Addition of free ionic Cu(I) or Cu(I)–diglutathione to apohemocyanin results in an instantaneous 10% decrease of the protein's intrinsic fluorescence at 331 nm (results not shown). The immediate decrease is followed by a very slow, but much larger decrease (35%) of the protein fluorescence which is caused by the quenching of the fluorescence emission of an active-site tryptophan following incorporation of Cu(I) into the oxygen binding site (see next paragraph and Discussion). HPLC analysis does not show any copper bound to hemocyanin (Hcy) until 5 min after addition of Cu(I). It seems, therefore, that the initial binding of Cu(I) is fast and reversible, which is followed by a slow insertion of copper into the active site. Ultrafiltration experiments, carried out with apohemocyanin incubated with Cu(I)–GSH for only 1 min to prevent internalization of Cu(I), show that a plot of [Cu]/[Hcy] vs free [Cu(I)] is a simple saturation curve, which becomes saturated at 1 Cu/Hcy (Figure 2). A Scatchard plot (Scatchard, 1949) of this dataset shows 1.02 ± 0.09 binding sites for copper per hemocyanin subunit with an apparent, conditional dissociation constant of 2.14 ± 0.59 μM. Analysis of the time course of copper binding to apohemocyanin after size-exclusion HPLC shows that 50% of the copper (one copper) is incorporated into the protein matrix after 10 min of incubation (Figure 5). This incorporation is not accompanied by restoration of oxygen or carbon monoxide binding capacity, and does not lead to quenching of the fluorescence of the active-site tryptophan (Figure 5). The incorporation of the first copper therefore represents internalization of the surface-bound metal into the "copper channel". The movement of this copper to the active site is slow, and constitutes the rate-limiting step in the Cu(I)–GSH-mediated reconstitution process. This issue will be further addressed under Discussion.

Formation of CO and O_2 Binding Sites. The time course of restoration of oxygen binding by apohemocyanin, as measured by UV spectroscopy, is shown in Figure 3. The slow changes in fluorescence emission that accompany incorporation of copper into the active site, measured at 331 nm, and formation of CO binding binuclear copper sites, measured at 580 nm, are shown in Figure 4. To distinguish between the fluorescence quenching at 331 nm due to the incorporation of copper into the active site and the subsequent, additional, quenching that results from the binding of CO to the restored active sites, two reconstitution experiments were carried out, one in a nitrogen atmosphere and the second in CO. A linear relationship was found between the quenching at 331 nm in nitrogen and the Cu(I)–CO fluorescence at 580 nm in CO (R^2 = 93.71%). Similarly, the decrease in fluorescence at 331 nm

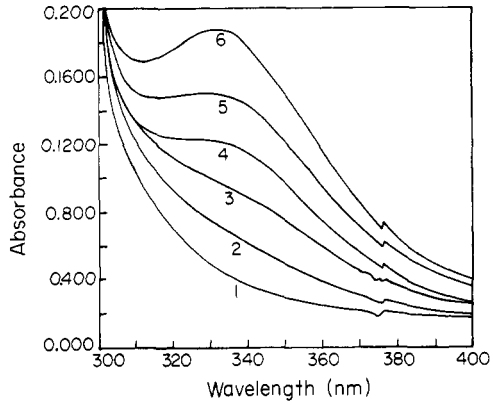

FIGURE 3: Reconstitution of the oxygen binding capacity of copper-free hemocyanin by Cu(I)–glutathione in 10 mM Bis-Tris, pH 6.0, 5 mM $CaCl_2$, and 0.5 M NaCl. A solution of 10 μM apohemocyanin was incubated in a CO atmosphere with 40 μM Cu(I) in the presence of a 5-fold excess of glutathione over copper. Subsamples were taken as a function of time, and absorbance spectra were recorded after displacement of CO by pure oxygen and increasing the pH from 6 to 8.5: (1) 1, (2) 30, (3) 60, (4), 120, (5) 180, and (6) 600 min.

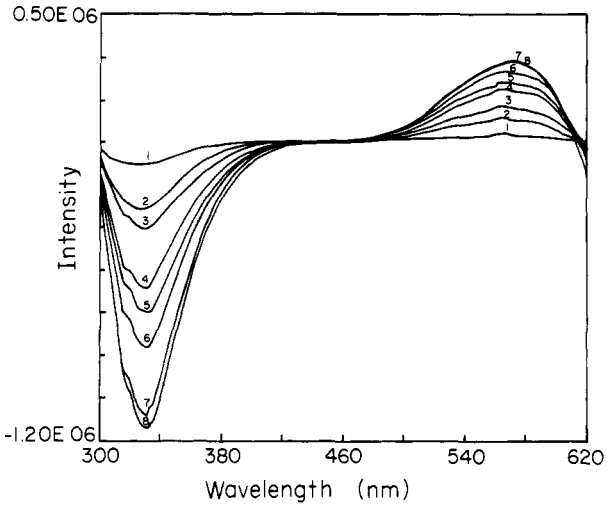

FIGURE 4: Reconstitution of binuclear copper sites and carbon monoxide binding capacity of copper-free hemocyanin by Cu(I)–glutathione. Conditions as described in Figure 3. Fluorescence emission spectra were recorded as a function of time, and are presented as difference spectra. The fluorescence quenching at 331 nm is due to incorporation of copper into the active sites and subsequent binding of CO. The fluorescence emission signal at 580 nm represents binding of CO to restored binuclear copper sites: (1) 10, (2) 30, (3) 60, (4) 90, (5) 180, (6) 250, (7) 300, and (8) 600 min.

in CO was linearly dependent both on the fluorescence quenching at 331 nm in nitrogen and on the Cu(I)–CO fluorescence at 580 nm, with R^2 values of 95.79 and 97.80%, respectively. This indicates that copper, once incorporated into the active site, can combine with CO. Since the copper–CO fluorescence signal at 580 nm requires the presence of binuclear copper sites (Finazzi-Agro et al., 1982; Zolla et al., 1984), and since the incorporation of the first copper into the active site is a slow process, this implies that insertion of the first copper into the active site greatly enhances the rate at which the second copper is inserted. The fractional saturation of hemocyanin with oxygen and CO, derived from the data presented in Figures 3 and 4, is shown in Figure 5. The rate of conversion of deoxy sites into CO and oxygen binding sites, calculated from the data presented in Figure 5, is kinetically a first-order process with a reaction half-time of 95 ± 3 min for CO and 102 ± 9 min for oxygen. Restoration of CO and oxygen binding is thus kinetically identical with an overall reaction half-time of 100 ± 5 min. The rate was found to be

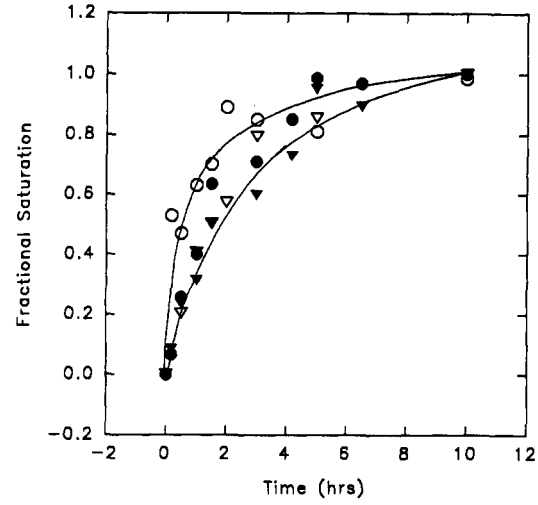

FIGURE 5: Fractional saturation of reconstituted hemocyanin with copper, oxygen, and CO. Conditions were as in Figure 3. The CO and O_2 data are derived from data presented in Figures 3 and 4. The fractional saturation with copper was determined by size-exclusion HPLC and atomic absorption spectroscopy, and by fluorescence spectroscopy: (○) copper (from HPLC); (▼) copper (from fluorescence quenching at 331 nm); (▽) oxygen; (●) CO.

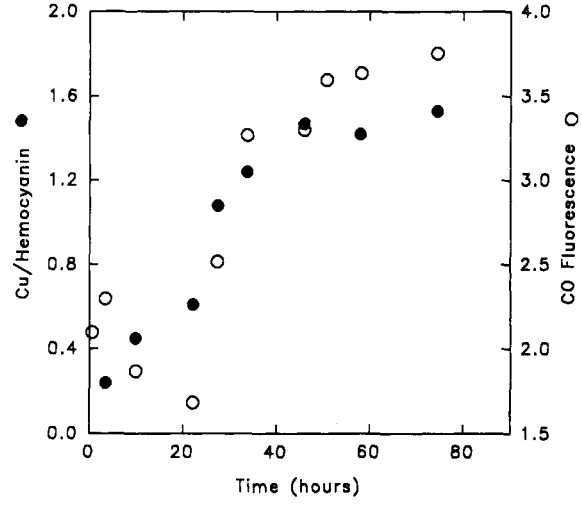

FIGURE 6: Reconstitution of copper-free hemocyanin with Cu(I)–glutathione in 10 mM Bis-Tris, pH 7.5, and 5 mM $CaCl_2$. 10 μM apohemocyanin was incubated with 40 μM Cu(I) and 200 μM glutathione in a CO atmosphere. The amount of copper bound was measured by size-exclusion HPLC and atomic absorption spectroscopy. CO binding was measured by fluorescence spectroscopy. (●) Copper; (○) CO.

dependent on the age of the apoprotein. For example, after 4 days, the half-time had increased to 319 ± 5 min.

pH Dependence of Reconstitution. The time course of reconstitution at pH 7.5 is shown in Figure 6. Incorporation of copper is a very slow process, and occurs mainly between 10 and 74 h of incubation. The maximum amount of copper bound to hemocyanin is 1.5 Cu/Hcy. The CO fluorescence signal increases between 32 and 74 h. Restoration of oxygen binding capacity is minimal. The active sites can combine with CO, but not with oxygen. Size-exclusion HPLC of apohemocyanin oligomers or subunits incubated with Cu(I)–diglutathione at pH 8.5 for extended periods of time shows that the apoprotein is incapable of incorporating Cu(I) under those conditions.

Oxygen Binding and Oxygen Dissociation Experiments. To probe the conformation of the active site, oxygen binding equilibrium experiments and oxygen dissociation kinetic experiments were carried at the three conditions used for re-

Table I: Oxygen Binding Properties of *Homarus* Hemocyanin

pH	*p*50 (mmHg)	*n*50	k_{off} (s^{-1})
6.0 (NaCl)[a]	275	1.33	>1200
7.5[a]	107	2.78	>1200
8.5	13.5	3.20	31 → 200
8.5	46	1.1	77

[a] Oxygen dissociation is complete within the dead time of the stopped-flow apparatus (2.5 ms).

constitution experiments. The results are summarized in Table I. Oxygen affinity of lobster hemocyanin is extremely low at pH 6, still very low at pH 7.5, and moderately high at pH 8.5. The decrease in oxygen affinity with decreasing pH is kinetically reflected in a large increase of the oxygen dissociation rate.

Sedimentation Analysis. Sedimentation velocity experiments were carried out to determine the aggregation state of hemocyanin under our experimental conditions. At both pH 6.0 and pH 7.5, the majority of the hemocyanin had a sedimentation coefficient of 25 S, corresponding to dodecamers. At pH 8.5, both dodecamers and hexamers were present.

Discussion

Cu(I)–GSH may be the source of Cu(I) for incorporation into apometallothionein and aposuperoxide dismutase (Freedman et al., 1989; Ciriolo et al., 1990; Steinkuhler et al., 1991), and it has been proposed that GSH forms a complex with CuMT (Freedman, 1989). GSH binds to CuMT from the American lobster and can displace Cu(I) from a specific isoform of this protein (Brouwer et al., 1992a; Brouwer & Brouwer-Hoexum, 1991). In vivo magnetic resonance microscopy suggests that copper accumulated in the hepatopancreas of the blue crab is diamagnetic Cu(I) (Brouwer et al., 1992b). Copper in both MT and hemocyanin occurs in the Cu(I) oxidation state, and their apoproteins can only be reconstituted with Cu(I) (Konings et al., 1969; Nielson et al., 1985). GSH is a likely source for maintaining the intracellular pool of free copper in the Cu(I) oxidation state (Freedman et al., 1989; Brouwer & Brouwer-Hoexum, 1991). The Cu(I)–GSH complex is very stable, even in air, whereas Cu(I)–cysteine is unstable (Ciriolo et al., 1990). These data suggest that GSH plays an important role in the biochemistry of copper regulation and in the activation of copper-dependent proteins. The results reported here further clarify this function of GSH.

We have previously shown that GSH coelutes with CuMT in a Sephadex profile of an aqueous extract from the hepatopancreas of the lobster (Brouwer & Brouwer-Hoexum, 1991). We have now established conditions that show the presence of Cu(I)–GSH (Figure 1). The GSH/Cu ratio of the low molecular weight peak (Figure 1, fraction 75) is much less than 2, indicating the presence of ligands other than GSH for copper. Preliminary data suggest that a Cys-Gly dipeptide, possibly derived from breakdown of GSH, is also present in these fractions (Brouwer & Brouwer-Hoexum, 1991; Brouwer et al., 1992a). Further characterization of this copper complex is under way.

Reconstitution of the binuclear copper site in apohemocyanin by Cu(I)–GSH can, as a first approximation, be described by the following steps: (1) binding of free ionic Cu(I) [or Cu(I)–diglutathione] to external site(s) on the hemocyanin molecule; (2) internalization of copper and movement of the metal through the "copper channel" in the hemocyanin protein matrix; (3) binding of the first Cu(I) to the histidines in the active site to form a mononuclear copper site; (4) binding of the second copper to the external Cu(I) site, with subsequent movement through the copper channel followed by formation of the binuclear copper site; and (5) rearrangement of the newly-formed oxygen binding pocket into the biologically active conformation. Data obtained during this study that shed light on these steps are discussed in the following section.

Addition of Cu(I) or Cu(I)–GSH to either apo- or holohemocyanin results in an instantaneous 10% decrease of hemocyanin's fluorescence emission at 331 nm, indicating binding of Cu(I) to the protein. In the case of apohemocyanin, this fast fluorescence change is followed by a much slower and larger (35%) decrease of the fluorescence signal due to incorporation of copper into the active site (Engel & Brouwer, 1987). The latter signal is most likely associated with Trp-197, which is only 5.7 Å away from CuA, and conserved in all arthropod hemocyanins sequenced so far (Linzen et al., 1985; Volbeda, 1988). Size-exclusion HPLC showed that irreversible binding of Cu(I) to apohemocyanin was only evident after the protein and metal had been incubated for at least 5 min. Internalization of Cu(I) did not occur with holohemocyanin. We postulate that the instantaneous binding of Cu(I) is reversible and that, owing to the metal binding capacity of TSK HPLC columns, the equilibrium between copper and hemocyanin is disrupted during transport through the column matrix. Analysis of the reversible Cu(I) binding step by ultrafiltration experiments revealed the presence of 1 (1.02 ± 0.09) external binding site for Cu(I) per hemocyanin subunit, with an apparent K_{diss} of 1.67 ± 0.40 μM, in the presence of a 5-fold excess GSH over Cu(I). Measurement of GSH in filtrates and retentates obtained after ultrafiltration showed that GSH does not form a stable complex with hemocyanin. However, these measurements do not rule out the possibility that Cu(I)–GSH forms a transient complex with the protein which rapidly dissociates into hemocyanin–Cu(I) and GSH. To examine if reconstitution proceeds via free Cu(I) or Cu(I)–GSH, ultrafiltration and CO fluorescence experiments were carried out with apohemocyanin and a 20-fold excess of GSH over Cu(I). Under those conditions, both the amount of Cu(I) bound to hemocyanin and the rate of reconstitution of active sites were diminished with respect to experiments carried out at a 5-fold excess of GSH over Cu(I) (data not shown). These results strongly suggest that reconstitution does not involve the Cu(I)–GSH complex, but free ionic Cu(I).

The internalization of the first Cu(I) into apohemocyanin, and subsequent incorporation of the metal into the active site, occurs on two different time scales. Fifty percent (one copper) is irreversibly bound during the first 10 min of incubation. Binding of the first copper does not result in restoration of ligand binding capacity or fluorescence quenching of hemocyanin (Figure 5). The latter observation suggests that copper is not present in the active site, since reconstitution of mononuclear copper sites in arthropod hemocyanins results in 40% quenching of the hemocyanin fluorescence emission (Salvato et al., 1986; Beltramini et al., 1986). We conclude, therefore, that the binding of one Cu(I) during the first 10 min represents internalization of the surface-bound metal into the copper channel. Movement of the copper through the channel into the active site is a slow process, as judged from the time course of the quenching of the fluorescence emission that accompanies the incorporation of copper into the active site.

The next question that needs to be addressed concerns the rate at which the nonfunctional mononuclear copper sites are converted into functional binuclear copper sites. The linear relationship that we observed between the fluorescence quenching in a nitrogen atmosphere, caused by the incorporation of copper into the active site, and the fluorescence

increase due to the formation of [Cu(I)–Cu(I)–CO] in a CO atmosphere, clearly suggests that copper, once incorporated into the oxygen binding site, is functionally active, and thus binuclear. This in turn indicates that the formation of mononuclear copper sites greatly enhances the rate at which the second copper is inserted.

The data discussed above allow the reconstitution process to be summarized as follows: (1) Cu(I) binds to a specific, external site on the hemocyanin surface (entrance to the copper channel). (2) Cu(I) becomes internalized and moves slowly through the channel into the active site to form a mononuclear copper site. This is the rate-limiting step in the reconstitution process, and explains why reconstitution of CO and O_2 binding sites is kinetically a first-order process whose rate constant is independent of the protein concentration. (3) The second copper moves in quickly to give a functionally active, binuclear copper site. Whether copper insertion into apohemocyanin occurs in vivo after biosynthesis and folding of the protein or during translation is yet to be determined (see below).

The reconstitution process is extremely pH-dependent. Fluorescence measurements from pH 6 through pH 8.5 showed that the rapid quenching of the fluorescence emission caused by the binding of Cu(I) is independent of the pH. This indicates that the external binding site for Cu(I) is accessible at the pH values that were used in our investigations. UV titrations showed that the copper–thiolate charge transfer of the Cu(I)–GSH complex observed at 260 nm (Brouwer & Brouwer-Hoexum, 1991) was complete at GSH/Cu(I) = 2 at pH 6, 7.5, and 8.5, indicating that all of the Cu(I) at the three different pH values, and at a GSH/copper ratio of 5, was present as a Cu(I)–diglutathione complex. These measurements indicate that the decreased efficiency of reconstitution of apohemocyanin with increasing pH values is not due to loss of the external Cu(I) binding site or to a change in the chemistry of the Cu(I)–GSH complex.

At pH 8.5, no copper was incorporated into the protein at all, and at pH 7.5, incorporation was incomplete. The copper inserted at pH 7.5 could only combine with CO, a non-copper-bridging ligand, but not with the copper-bridging dioxygen molecule. This suggests that the binuclear copper site formed under those conditions is stereochemically distorted and can only accommodate a ligand that combines with one of the coppers. We have made similar observations in a previous study where reconstitution was carried out at pH 8 with a copper complex isolated from lobster hepatopancreas (Brouwer et al., 1989). Such observations suggest that the conformation of the active site is an important factor in the reconstitution process. To examine if the observed pH dependence could be correlated to the conformation of the active site, we determined the oxygen binding properties of the sites at the three conditions used for reconstitution experiments. The oxygen affinity of lobster hemocyanin at pH 6 was extremely low, and the oxygen dissociation was complete within the dead time (2.5 ms) of the stopped-flow apparatus (Table I). At pH 8.5, the hemocyanin had a moderately high affinity for oxygen, and the oxygen dissociation kinetics showed autocatalytic behavior; i.e., the oxygen dissociation rate increased from 31 to 200 s^{-1} during the oxygen unloading process. This behavior is characteristic of the R- to T-state transition of cooperative oxygen binding proteins (Van Driel et al., 1974). Subunits prepared from lobster hemocyanin at pH 8.5 showed simple first-order kinetics of oxygen dissociation with a rate constant of 77 s^{-1}. As is the case for the multisubunit hemocyanin at pH 8.5, the free subunits could not be reconstituted at this pH with Cu(I)–GSH (data not shown). The decrease in oxygen affinity with decreasing pH is kinetically reflected in a very large increase of the oxygen dissociation rate, which indicates that the structure of the binuclear copper pocket at pH 6 is loose and open and does not provide sufficient stabilizing forces for the oxygen molecule to remain bound. This may explain why incorporation of copper into lobster hemocyanin proceeds best at low pH, where the architecture of the active-site pocket is open and flexible enough to accommodate the coppers.

Our results show that Cu(I)–GSH is able to donate Cu(I) to copper-free hemocyanin in vitro. As noted, in vivo the Cu(I)–GSH complex may result from the interaction of GSH with a specific CuMT isoform (Brouwer & Brouwer-Hoexum, 1991). Our data and those of others (Steinkuhler, 1991; Freedman et al., 1989; Huber & Lerch, 1987) suggest that Cu(I)–GSH is an important form of low molecular weight, intracellular copper. During MT synthesis, this complex is the source of Cu(I) for formation of Cu(I)–MT. In addition, Cu(I)–GSH is the source of copper for the biosynthesis of other copper-dependent proteins. We consider it probable that, when the supply of copper in the diet is limited, the Cu(I)–GSH complex is formed at the expense of the Cu(I)–MT pool: Cu(I)–MT ↔ Cu(I)–GSH → Cu(I) for copper proteins. The irreversible incorporation of copper into copper-dependent proteins provides the driving force for the GSH-mediated removal of Cu(I) from Cu(I)–MT. These observations, and the results presented herein, provide additional evidence for the joint involvement of CuMT and GSH in the biosynthesis of copper proteins. To further evaluate the mechanism of intracellular copper incorporation into hemocyanin and the role of CuMT and GSH in this process, and to determine if copper incorporation occurs cotranslationally or posttranslationally, experiments are under way using radiolabeled copper and amino acids and blue crab hepatopancreas tissue in an in vitro culture system.

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Isolation of a Novel cDNA Encoding a Zinc-Finger Protein That Binds to Two Sites within the c-*myc* Promoter[†,‡]

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ABSTRACT: The ME1a1 and ME1a2 elements are cis-acting DNA sequences that exist at positions −46 and −85, respectively, within the P2 promoter of the c-*myc* gene. These elements are required for optimal transcription initiation from P2. The proteins that bind to these elements are identical or very similar. Here we have isolated a cDNA clone that encodes the carboxy-terminal 494 amino acids of the human ME1a1/ME1a2 factor. This factor is referred to as "ZF87" (zinc-finger protein, 87 kilodaltons). ZF87 specifically binds the ME1a1 element with higher affinity than the ME1a2 element. Western blot analysis indicates that the full-length protein has a relative molecular mass of approximately 87 000 daltons, and Northern blot analysis shows that it is encoded by a 5-kb transcript. ZF87 contains six zinc-finger domains, of the Cys_2-His_2 type, at the carboxy terminus of the protein. The protein also contains extended tracts of polyalanine.

Expression of the c-*myc* gene plays an important role in controlling cell proliferation (Cole, 1986). One mechanism that is involved in regulating c-*myc* expression occurs at the level of transcription initiation. However, to fully understand how transcription initiation is regulated, it is necessary to characterize the elements that constitute the c-*myc* promoter.

A majority of transcripts initate from the P2 promoter of the c-*myc* gene (Cole, 1986). While the region distal to the P2 promoter contains a number of enhancer and repressor elements (Avigan et al., 1990; Hay et al., 1987; Iguchi-Ariga et al.; 1988; Kakkis et al., 1989; Weisinger et al., 1988), the proximal region of the P2 promoter is composed of three

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[‡] The amino acid sequence reported in this paper has been submitted to GenBank under Accession Number J05371.
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