Specific Receptor for Ceruloplasmin in Membrane Fragments from Aortic and Heart Tissues†

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ABSTRACT: Membrane fragments from aortic and heart tissues of immature chicks were observed to bind highly purified, ^{125}I-labeled chick ceruloplasmin. The binding reaction exhibited a linear Scatchard plot for both tissues and showed for each an apparent dissociation constant (K_d) of about 10^{-8} M. On the basis of Scatchard analyses, aorta contained 1.5 pmol of receptors/mg of membrane protein, whereas receptors in the membranes from heart tissue were at least 5 times more dense. The binding of chick ceruloplasmin to aorta membranes was trypsin sensitive and neuraminidase insensitive, and showed both saturation and reversibility. Various sialoglycoproteins in 500 molar excess had very little effect on the binding. The asialo derivatives of these proteins likewise did not inhibit the binding. Human ceruloplasmin was found to bind very weakly to the chick membranes. Asialo chick ceruloplasmin bound with the same efficacy as native chick ceruloplasmin. Heat-denatured chick ceruloplasmin, however, was very ineffectual in displacing native ^{125}I-ceruloplasmin from the membranes. These studies provide the first evidence for a homologous membrane receptor for native ceruloplasmin in the plasma membranes of animal cells.

Ceruloplasmin, a serum copper protein, has received favorable attention as a possible transport agent for copper. The protein, synthesized in liver, is believed to transfer copper from liver to the tissues, ultimately to the intracellular metalloenzymes that require copper for function (Owen, 1965; Marceau & Aspin, 1973; Hsieh & Frieden, 1975; Terao & Owen, 1976; Linder & Moor 1977; Harris & DiSilvestro, 1981; Campbell et al., 1981). Alternatively, amino acid complexes of copper (Neumann & Silverberg, 1966; Neumann & Sass-Kortsak, 1967; Harris & Sass-Kortsak, 1967), serum albumin (Sarkar & Wigfield, 1968), and a tripeptide composed of glycine-histidine-lysine (Pickart & Thaler, 1980; Pickart et al., 1980) also may play a role in determining the cellular bioavailability of copper. The conclusion that ceruloplasmin is the major transport protein for copper must weigh contributions from the non-ceruloplasmin fractions of serum as well. The problem requires further clarification. Then too, the copper atoms bound to ceruloplasmin are associated with its oxidase activity toward ferrous ions (Curzon & O'Reilly, 1960; Osaki et al., 1966; Shokeir, 1972; Planas & Frieden, 1979; Williams et al., 1974), biogenic amines (Peisach & Levine, 1963; Frieden, 1979), and possibly oxygen radicals (Al-Timimi & Dormandy, 1977; Goldstein et al., 1982). It is hard to reconcile both oxidase activity (which requires an intact protein) and transport–delivery activity residing within the same protein.

In earlier work we found circumstantial evidence that links ceruloplasmin with the activity of lysyl oxidase, a copper metalloenzyme in connective tissue. Both are suppressed in a nutritional copper deficiency and both respond upward and rapidly to the administration of micromolar amounts of copper salts (Harris & DiSilvestro, 1981). The copper-induced activation of lysyl oxidase correlates with elevation of plasma ceruloplasmin levels. We have further determined that the activation mechanism involves the transfer of copper to newly synthesized protein in aortic tissue (Rayton & Harris, 1979). Further steps in the mechanism are not known, although recent studies have tended to discount the participation of metallothionein in the transfer of aortic copper intracellularly (Balthrop et al., 1982).

If ceruloplasmin functions in the transport and delivery of copper to lysyl oxidase, it should be possible to observe a specific interaction of ceruloplasmin with the membrane fraction of aortic tissue cells. Presumably, such an interaction would signify the presence of receptors for ceruloplasmin in the aortic cells. The present study represents a search for such putative receptors. For comparative purposes, we have also examined heart tissue membranes for ceruloplasmin receptors. The rapidly growing, immature chick is the model system for these studies.

Materials and Methods

Animals. Inbred male hybrid leghorn chicks were obtained on the day of hatching and housed at 28–32 °C in all-metal brooders (Petersime, Inc., Gettysburg, OH). Tap water and prepared diets were fed ad libitum as previously described (O'Dell et al., 1966).

Purification of Glycoproteins. Crystalline orosomucoid and fetuin were obtained from Sigma Chemical Co. and further purified on DEAE-Sephadex. The proteins were applied to separate 2.5 × 10 cm columns that had been equilibrated against 0.05 M NaH_2PO_4, pH 6.8. The absorbed proteins were eluted with a gradient of 0.0–0.8 M NaCl. The major protein fraction that emerged was collected and concentrated by ultrafiltration (Amicon membrane, Y-30).

To prepare the asialo derivatives, solutions of orosomucoid, fetuin, or chick ceruloplasmin (2 mg/mL) were incubated separately with 0.5 unit of *Clostridium perfringens* neuraminidase at 37 °C for 3 h. The Warren test was used to monitor the neuraminic acid released (Warren, 1959). The asialo derivatives were separated one at a time by gel filtration using Sephadex G-200 (2.5 × 25 cm column) in 0.03 M NaH_2PO_4, pH 7.2. Asialoorosomucoid and asialofetuin eluted as single peaks near the midpoint of elution volume. Asialoceruloplasmin appeared as a single peak in the void volume of the column. In all cases the single fraction with the highest

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absorbance at 280 nm was used in the studies.

Preparation of Aortic and Heart Membranes. Membranes were prepared by a modification of the method described by Chausmer et al. (1980). Eleven-day-old chicks were decapitated and the aortas removed as described by Harris (1976). Approximately 20–25 aortas (0.8–1.0 g) or five hearts (1.5 g) were placed in 10 mL of ice-cold homogenization buffer (0.25 M sucrose, 5 mM EDTA,[1] and 10 mM imidazole, pH 7.5). The tissue was homogenized with a Virtis 45 homogenizer at half-speed for 30 s in the cold. Adhering tissue was returned to the suspension, and homogenization was continued an additional 20 s. The homogenate was poured into a 30-mL Corex centrifuge tube. The probe and container were washed with 10 mL of buffer, and this was poured into the same Corex tube and centrifuged at 1000*g* for 15 min in a Sorvall Model RC-5B centrifuge. The resulting supernatant was centrifuged at 78000*g* for 45 min with a Beckman Type 30 rotor in a Beckman Model L2-65B Ultracentrifuge. The resulting pellets from one tube containing heart tissue or two or three tubes containing aorta tissue were suspended in 2 mL of 1 mM imidazole buffer, pH 7.5, and rehomogenized in a Dounce tissue homogenizer with 15 strokes of a tight-fitting pestle. The homogenate was layered on a 10-mL, 15–50% (w/v) linear sucrose density gradient in 1 mM imidazole buffer and centrifuged at 100000*g* for 14–16 h at 4 °C by using a Beckman SW 40 rotor. One-milliliter fractions of the gradient were obtained by puncturing the bottom of the centrifuge tube with a 20-gauge needle and collecting the drops (14 drops/mL). Five milliliters of the imidazole buffer was added, and each fraction was placed in a Beckman 50 Ti rotor and centrifuged at 100000*g* for 45 min. The supernatant was decanted, and the pellets were resuspended in 2 mL of buffer. The protein concentration in each fraction was determined by the method of Lowry et al. (1951) by using bovine serum albumin as a reference standard. The 5′-nucleotidase activity was tested by the method of Solyom & Trams (1972). The activity served as a marker for the plasma membranes. The appropriate fractions were pooled, centrifuged at 100000*g* for 30 min, and adjusted to an estimated concentration of 100–300 μg/mL with 1 mM imidazole buffer, pH 7.5. An accurate protein concentration was used to determine quantities used in each binding study.

Purification and Iodination of Chick Ceruloplasmin. Pooled chick serum with trypsin inhibitor (ε-aminocaproic acid) added was obtained from PelFreeze Inc., Rogers, AR. Chick ceruloplasmin was purified to homogeneity by the method of R. A. DiSilvestro and E. D. Harris (unpublished data). Briefly, the purification scheme used ethanol–$CHCl_3$ precipitation followed by chromatography on DEAE-Sephadex. Further purification on Sephadex G-200 and CM-cellulose yielded a blue protein with strong oxidase activity toward *p*-phenylenediamine and ferrous ions. This protein had an A_{610}/A_{280} ratio of 0.07 indicative of very high purity (Starcher & Hill, 1966). Purity was established further by noting the protein showed one band on nondenaturing polyacrylamide gels and one arc on Grabar immunoelectrophoresis. With sodium dodecyl sulfate present, faint traces of lower molecular weight material were detectable with polyacrylamide gels. Such dissociation in denaturing gels reflects intrinsic instability of ceruloplasmin and is not indicative of the presence of contaminating proteins (Kingston et al., 1977). Two different measurements suggested ceruloplasmin from chick had a molecular weight of 132000–134000. We used 133000 in our calculations. The purified chick ceruloplasmin was iodinated with ^{125}I (Amersham Corp.; specific activity 17 Ci/mg) enzymatically by the lactoperoxidase Enzymobeads (Bio-Rad Laboratories) by using the procedure recommended by Bio-Rad. A clinical centrifuge was used to separate the beads, and the supernatant was applied to a Sephadex G-50 column (0.5 cm × 10 cm) equilibrated against 0.03 M NaH_2PO_4 with 0.25% w/v bovine serum albumin (highest purity; Boerhinger-Mannheim) to the counteract nonspecific absorption to the column. Components appearing in the void volume fractions were used as the source of labeled ceruloplasmin in this study. Specific activities for each preparation were calculated on the basis of percent incorporation as determined from the chromatography.

Binding Studies. A Scatchard analysis was used to determine binding constants and receptor concentrations (Scatchard, 1949). Aorta membranes (250–500 μL suspension containing 50–150 μg of protein) were incubated with 0.5–0.9 pmol of ^{125}I-ceruloplasmin (specific activity 2500–3500 cpm/fmol) and varying amounts of unlabeled ceruloplasmin ranging from 0.5 to 100 pmol. Nonspecific binding was determined by measuring the counts bound in the presence of 300–500 pmol of unlabeled ceruloplasmin. All tubes were brought to 1 mL by adding the appropriate amount of incubation buffer (100 mM HEPES, 120 mM NaCl, 15 mM sodium acetate, 10 mM glucose, 1.2 mM $MgSO_4$, and 1 mM EDTA, pH 7.5). They were incubated at 4 °C for 14–16 h. Quadruplicate aliquots (200 μL) from each tube were centrifuged in a Beckman Microfuge 12 at 12000*g* for 15 min. The supernatant was removed by aspiration, 300 μL of incubation buffer was added, and the tubes were centrifuged again. After aspiration, a 5-mm section of the tip in a 12 × 75 mm test tube was placed in a Beckman Gamma 5500 system with a ^{125}I-isoSet. The radioactivity in the tips determined the counts bound in 200 μL of the original reaction mixture. Free radioactivity was measured as the difference between total (added) and bound. Subtracting the nonspecific binding from the counts bound resulted in net counts bound. Both net bound and free counts were converted to ceruloplasmin concentration by using the appropriate ^{125}I-ceruloplasmin specific activity. Bound was expressed as picomoles per milligram of protein and the free as nanomolar ceruloplasmin. The kinetic parameters were determined by a least-squares fit of a plot of bound/free vs. bound where the slope represents $-1/K_d$ and the x intercept is the receptor concentration.

Results

Preparation of Membranes and ^{125}I-Ceruloplasmin. The membrane fragments used in this study were prepared from homogenates of whole aorta and heart tissues. The specific membrane fraction equilibrated in a sucrose gradient at a density of 1.12–1.17 g/cm^3, the range expected for plasma membranes (Emmelot & Bos, 1970). Only those fractions which showed appreciable amounts of the marker 5′-nucleotidase activity were used (Figure 1). It can be seen that this activity did not coincide with a heavier major protein fraction in the gradient, thus giving some assurance that the lighter plasma membrane fractions were major components in the binding assays to follow.

The iodination of highly purified chick ceruloplasmin resulted in about 70% incorporation of the added ^{125}I to the protein. The unincorporated radioactivity was readily separated by means of a Sephadex G-50 column run in the presence of 0.25% (w/v) bovine serum albumin (Figure 2).

Scatchard Analysis of the Binding. The binding of the ^{125}I-ceruloplasmin to the membrane fragments varied with the

[1] Abbreviations: EDTA, ethylenediaminetetracetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

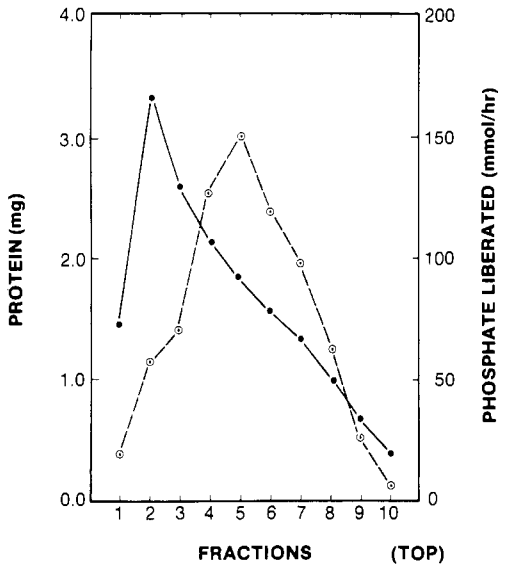

FIGURE 1: Preparation of plasma membranes by sucrose-density gradient centrifugation. Membranes were obtained from 97 chick aortas. Protein (●) and 5′-nucleotidase activity (⊙) were determined on each fraction. Fractions 3–7 were pooled for binding studies.

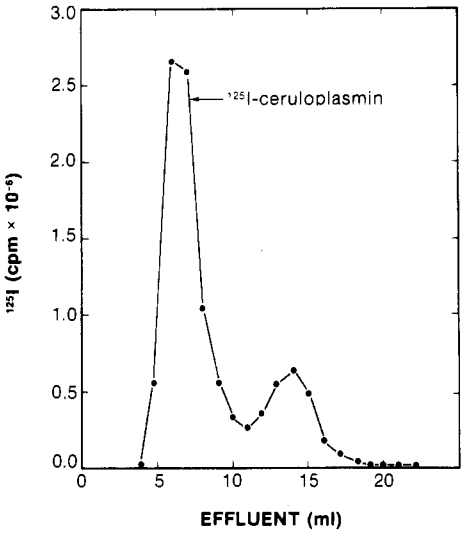

FIGURE 2: Iodination of chick ceruloplasmin. Unincorporated radioactivity was separated from bound by means of a Sephadex G-50 column. In this preparation a 73.8% incorporation of ^{125}I was observed resulting in a specific activity of 2.94×10^3 cpm/fmol.

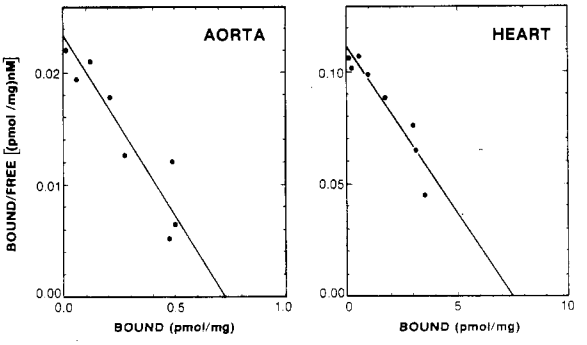

FIGURE 3: Scatchard analyses of the binding of chick ceruloplasmin to aorta and heart membranes. The K_d values were 3.28×10^{-8} M for aorta and 6.59×10^{-8} M for heart. Receptor concentrations were estimated to be 0.72 pmol/mg of protein for aorta and 7.32 pmol/mg of protein for heart.

specific activity of the protein. More important, the data for the binding gave rise to a linear Scatchard plot (Figure 3). Both aorta and heart membranes showed linearity. The slope

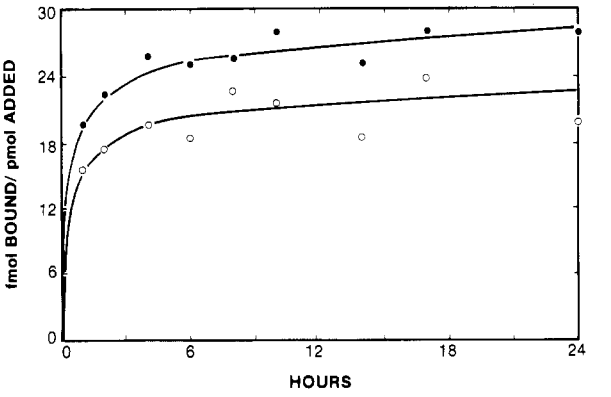

FIGURE 4: Time-course binding of ^{125}I-ceruloplasmin to aorta plasma membranes. Shown are total (●) and nonspecific (○) binding.

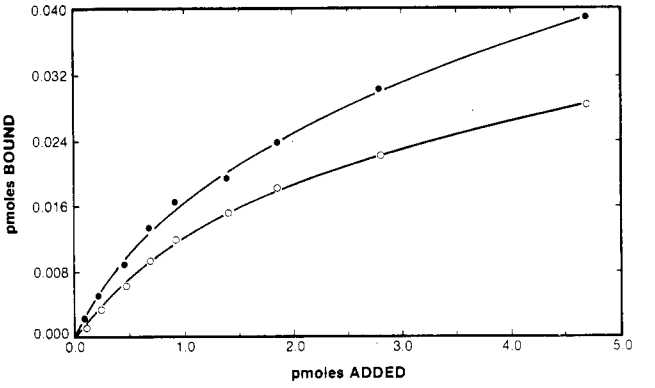

FIGURE 5: Binding of ^{125}I-ceruloplasmin as a function of ceruloplasmin concentration. Nonspecific binding for each point was determined by adding a 500 molar excess of unlabeled ceruloplasmin. Total (●) and nonspecific (○) binding.

of the plots for each tissue, a direct measure of the disassociation constant (K_d), approximated a value of 10^{-8} M, suggesting similar binding affinities to both sources of membrane. However, the density of the receptors (determined by the intercept on the x axis) appeared to be 10 times greater in heart tissue than in aorta (7.3 vs. 0.72 pmol/mg of protein). A more extensive analysis of the aorta membrane binding using nine separate Scatchard analyses showed an average disassociation constant of 45 ± 7.3 nM and a receptor concentration of 1.5 pmol/mg of membrane protein.[2]

Kinetic Analysis of the Binding. A number of studies were performed to characterize the binding of ceruloplasmin to the receptor. Binding equilibrium was achieved fairly rapidly, usually within 6–8 h (Figure 4). Beyond 10 h both the total and nonspecific binding tended to reach plateaus suggesting that the specific binding over this period was constant. The binding approached saturation when the ceruloplasmin in the assay medium was about 5 pmol (Figure 5). In binding assays of this type one must judge saturation when the Δ values for nonspecific binding and total binding are the same. Nonspecific binding will increase each time protein is added to the assay. When this increase parallels the increase in total binding, saturation is apparent. It can be assumed on this basis that the level of ceruloplasmin used in the Scatchard analyses and other binding studies (0.5–1.0 pmol) was well below the level of saturation. Binding thus was dependent on the ceruloplasmin concentration. As seen in Figure 6, adding an excess of cold ceruloplasmin to an equilibrated system resulted in a rapid displacement of the radioactivity from the receptor, reducing the total bound radioactivity to the level of the nonspecific. These data support the reversibility of the binding,

[2] Individual values will be furnished to interested readers on request.

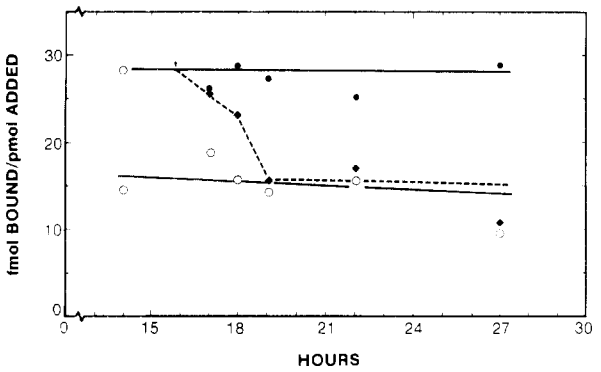

FIGURE 6: Reversibility of the binding of ^{125}I-ceruloplasmin to aorta membranes. Binding was allowed to proceed for 14–16 h. Arrow shows time point when a 500 molar excess of unlabeled ceruloplasmin was added. Total binding (●), nonspecific binding (○), and binding observed after addition of unlabeled (♦ --- ♦) are shown.

Table I: Efficacy of Various Sialo- and Asialoglycoproteins To Displace ^{125}I-Labeled Ceruloplasmin from Aortic Membranes

protein added[a]	fmol bound/ pmol added[b]	% bound
^{125}I-ceruloplasmin (chick)	26.33 ± 4.13 (n = 24)	100
orosomucoid	24.11 ± 4.48 (n = 20)	91.6
fetuin	24.45 ± 4.05 (n = 20)	92.9
transferrin	24.14 ± 5.06 (n = 20)	91.7
ceruloplasmin (human)	23.77 ± 0.98 (n = 4)	90.3
ceruloplasmin (chick)	16.85 ± 3.33 (n = 24)	64.0
asialoorosomucoid	29.19 ± 5.64 (n = 16)	110.0
asialofetuin	29.48 ± 5.52 (n = 16)	112.0
asialoceruloplasmin (chick)	18.04 ± 3.13 (n = 8)	68.5

[a] All proteins were added in a 500–1000 mol excess. Incubation lasted 24 h and was at 4 °C. [b] Data ± SEM.

a condition that is obligatory to meaningful interpretation of the Scatchard analysis (Scatchard, 1949).

Characterization of the Binding. Further studies were aimed at characterizing the specificity of the binding and the chemical nature of the receptor. Table I reports total binding and compares the efficacy of various proteins to obtain the level of nonspecific binding that is achieved when excess chick ceruloplasmin is added. As shown in the table purified orosomucoid, fetuin, and transferrin (all from a human source) were only partially effective in displacing the ^{125}I-ceruloplasmin (chick) from the binding sites on the membranes. In fact one could calculate that all intact proteins tested were only about one-fifth as effective as chick ceruloplasmin in competing for the membrane site. The most effective displacer was chick ceruloplasmin. Surprisingly, human ceruloplasmin was likewise quite inferior in displacing the chick protein. That observation suggests that there are structural dissimilarities in the ceruloplasmins from human and chick. Freeing the sialic acid from orosomucoid and fetuin did not enhance their displacement effectiveness. In fact the desialylated proteins caused more ^{125}I-ceruloplasmin to bind. The meaning of that observation has not been clarified. Asialo chick ceruloplasmin showed approximately the same competitive binding efficacy as native chick ceruloplasmin.

With regard to the membranes themselves, a brief incubation with neuraminidase (0.2 unit/mL) had no effect on the binding of chick ceruloplasmin. Digesting the membranes with trypsin (0.2 mg/mL), however, completely eliminated all traces of the binding activity. Clearly, the ceruloplasmin receptor has a protein character and appears not to require sialic acid as part of the recognition or binding site.

When native chick ceruloplasmin was heated (80 °C, 60 min) either before or after iodination, the protein showed considerably less ability either to bind to the membranes or to displace native ceruloplasmin in a competitive binding assay (Table II). Note that all possible combinations of heat-treated (denatured) and non-heat-treated (native) were tested. The heat treatment did not totally eliminate the binding since native ceruloplasmin in excess showed some displacement of the heated, radioactive protein. However, heat-treated, unlabeled ceruloplasmin in excess did not displace any radioactive protein, native or denatured. Note that more ^{125}I-ceruloplasmin bound when there was an excess of heat-denatured protein in the assay medium. These data provide supporting evidence for the tertiary structure of ceruloplasmin or a specific arrangement of groups on the protein surface as being a factor in the binding.

Table II: Binding of Heat-Denatured Ceruloplasmin to Aortic Membranes[a]

unlabeled ceruloplasmin added	^{125}I-labeled ceruloplasmin added: heat treated[b] fmol bound/ pmol added (mean ± SD)	% of control	untreated fmol bound/ pmol added (mean ± SD)	% of control
none (control)	18.55 ± 0.06	100	23.33 ± 0.06	100
untreated	15.46 ± 0.13	83.3	17.50 ± 0.12	75.0
heat treated	20.95 ± 0.06	112.9	26.92 ± 0.15	115.4

[a] Data ± SEM. [b] Heated at 80 °C, 60 min prior to assay.

Discussion

A specific receptor for ceruloplasmin exists in the plasma membrane fraction from immature chick aorta and heart tissues. The receptor appears to be a protein and to be distributed more abundantly in heart tissue than aorta. Because these studies used homogenates of whole heart and aorta to prepare the membranes, the precise cell type giving rise to the receptors cannot be determined at this time. Since membranes came from two tissue sources and both showed the presence of receptors, it can be assumed that ceruloplasmin receptors may occur in many cell types and that a search for such receptors in other tissues seems warranted.

A major difficulty with these studies has been the high level of nonspecific binding. We do not feel, however, that this should discourage future efforts at locating receptors for ceruloplasm in other tissues. Holmberg & Laurell (1948) noted that purification of ceruloplasmin was made more difficult by the tendency of the protein to absorb on other proteins. In the present studies molecular association was kept to a minimum by using very dilute solutions of ceruloplasmin (less than 1.0 nmol) in assays. In all cases the binding for total was statistically greater than nonspecific. It is also important to note that other facets of the binding activity such as approach to saturation, reversibility, and linear Scatchard plots were all favorable and indicative of specific bimolecular interaction.

Previous studies have shown that the copper concentration in immature chick serum is about 0.2–0.3 μg/mL (Harris & DiSilvestro, 1981). Assuming that 85% of this copper is bound to ceruloplasmin (Bingley & Dick, 1969) and that the metal comprises 0.2% of the weight of chick ceruloplasmin (Starcher & Hill, 1966; DiSilvestro, 1982), one can estimate a range 0.6–1.0 μM for this 133 000-dalton protein in chick serum (DiSilvestro, 1982). Thus, the K_d for the binding of ceruloplasmin to both aorta and heart receptors is well within the range of physiological significance in this species.

Receptors for asialo and asialylgalacto glycoproteins have been found in the membrane fraction of hepatocytes (Ashwell

& Morell, 1974; Stockert et al., 1976). These receptors require calcium ions for the binding and appear to recognize only structurally modified glycoproteins (Pricer & Ashwell, 1971). Such receptors account for the rapid removal of asialoceruloplasmin from serum (Morell et al., 1968). Chick hepatocytes also contain receptors that recognize modified glycoproteins bearing exposed *N*-acetylglucosamine residues (Kawasaki & Ashwell, 1977). In the present study it was noted that the binding of chick ceruloplasmin occurred in the presence of EDTA, suggesting no need for calcium or any divalent cation, and that desialylation had very little effect on the binding. Further, a series of native and disialylated serum glycoproteins did not compete with the ceruloplasmin for the binding sites. It would appear that the receptors in aorta membranes are not the same as those in hepatocytes. Rather these receptors recognize the intact, sialylated protein and indeed may be specific for just this one protein.

It is perhaps of greater interest to draw comparisons between the chick receptor for ceruloplasmin and the well-characterized transferrin receptor that mediates the transport of iron into cells. Both receptors are trypsin sensitive and show relative specificity for the proteins they bind. Like the chick receptor, the transferrin receptor is resistant to destruction by neuraminidase treatment (Hemmaplardh & Morgan, 1976). Its major recognition site appears to be found in the protein-bound *N*-asparagine-linked, mannose-rich oligosaccharide chains (Newman et al., 1982). The transferrin receptor is known to occur in nucleated red cells from chick embryos (Williams & Woodworth, 1973). Young & Aisen (1980) reported an association constant of 1.62×10^7 M^{-1} for the high-affinity transferrin site on rat hepatocytes. The present study showed a disassociation constant of 4.5×10^{-8} M, which corresponds to an association constant of 2.2×10^7 M^{-1} for the ceruloplasmin receptor. This value thus compares favorably with the hepatocyte transferrin receptor. Further, it closely approximates the value of 4.1×10^7 M^{-1} reported for the transferrin receptor in rabbit retuculocytes (van Bockxmeer et al., 1978). In this study it was noted that transferrin did not impede the binding of ceruloplasmin to the membrane fragments. Hence, it seems safe to assume that although the receptors for ceruloplasmin and transferrin approximate the same binding affinity for their respective proteins, they are not one in the same receptor.

Presently, the mechanism for transporting copper from the liver to peripheral tissue enzymes has not been clarified fully. This study supports a specific interaction between ceruloplasmin and the plasma membranes of aorta and heart cells. Hsieh & Frieden (1975) showed that injections of ceruloplasmin intravenously effectively restored the cytochrome *c* oxidase activity in heart and other tissues of copper-depleted rats. Subsequent work by Linder & Moor (1977) confirmed that heart tissue adeptly incorporated ceruloplasmin intracellularly. In an extension of this work Campbell et al. (1981) found that ceruloplasmin-bound copper as opposed to albumin-bound copper was a far better source of intracellular copper for a number of organs including heart. Implicit in these studies, but never tested, was that for the ceruloplasmin to transfer copper across the membrane of cells required a specific interaction of ceruloplasmin with the membrane surface. The present study, taken in this light, adds further support to ceruloplasmin being a transport protein for copper, at least in aorta and heart. Moreover, the finding of more receptors in heart tissue when compared to aorta could reflect the greater concentration of mitochondria in heart tissue and the correspondingly greater need for copper.

On the other hand, observing an interaction of ceruloplasmin with the cell membrane could conceivably signal other important physiological events other than copper delivery. For instance, protection of the cell membrane from peroxidations (Al-Timini & Dormandy, 1977) or superoxide radical scavaging at the membrane surface (Goldstein et al., 1982) have been attributed to a nontransport function of ceruloplasmin. To assert that binding relates only to copper delivery is certainly premature at this stage of our understanding. Clearly what is needed now is more insight into the metabolic events that ensue once binding to the membrane has occurred. Those data in turn must come from studies using whole cells and not membrane fragments. We must conclude, therefore, that the physiological significance of the binding activity remains to be determined.

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Study of Hydrogen Exchange in Hemoglobin as a Function of Fractional Ligand Saturation†

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ABSTRACT: We have studied the hydrogen-exchange kinetics of hemoglobin A_0 as a function of ligand, CO, saturation. In the noncooperative system, azide binding to methemoglobin, the alterations in exchange kinetics are proportional to the average degree of ligation. However, in the case of CO binding to deoxyhemoglobin the changes in hydrogen-exchange pattern run ahead of the degree of ligation. The data can be best fitted assuming that all the liganded species, regardless of the number of ligands, show the same exchange properties. This two-state behavior must be the consequence of the fact that all the conformational changes leading to increased solvent accessibility take place when the first ligand is bound. Studies of the effect of pH changes and carbamoylation on the relationship between ligand binding and hydrogen exchange show that the observed differences of hydrogen exchange between deoxy and the liganded state are linked to the alkaline Bohr effect and to the state of the α-N-termini. As a consequence, at pH 9 despite a highly cooperative ligand binding isotherm the differences in hydrogen exchange between the deoxy and fully liganded species have vanished. We have to conclude that the hydrogen exchange is mirroring only the first part of the overall R to T transition. In all the experiments with pH changes and carbamoylation it is the liganded form that shows changes becoming more like the deoxy state. This is not consistent with a model where ligand binding removes a structural restriction in the less accessible deoxy state.

The cooperative binding of ligands such as oxygen and carbon monoxide to ferrohemoglobin is characterized by a sigmoidal binding isotherm and a large difference in the first and fourth binding constants (Adair constants). This cooperative binding behavior has been a central area of research in hemoglobin chemistry. The two-state thermodynamic model of Monod–Wyman–Changeux (1965) and the X-ray structural studies of Perutz and others have been both popular and instrumental in attempting to describe the sequential binding of ligands to ferrohemoglobin.

Unfortunately, direct structural studies and thermodynamic anlysis of the partially liganded intermediates are difficult due to their transient nature as dictated by the binding equilibria. Therefore, few studies of ligand-induced conformational changes have been done as a function of ligand saturation, and the results have been somewhat contradictory. Early studies by spin-label methods (Ogawa & McConnell, 1967; Ogata & McConnell, 1972, 1971) and by cicular dichroism (Simon & Cantor, 1969) indicated that conformational changes were linear with respect to fractional saturation. More recently, kinetic rebinding measurements following flash photolysis of the carboxyhemoglobin complex have shown that the conformational change lags behind the fractional change in ligation (Sawicki & Gibson, 1978). Finally, several recent studies by a variety of physical methods have revealed significant structural changes which occur with the binding of the first ligand at physiological pH. These observations are as follows: the thiol reactivity of the β-93 SH group runs well

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