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CERULOPLASMIN RECEPTORS OF ERYTHROCYTES

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Summary: Mammalian erythrocytes have been shown to bind ^{125}I labeled ceruloplasmin. Binding was reversible and specific. Scatchard analysis yielded linear plots with a Kd of approximately 5nM. The binding site appeared to be a protein located on the cell surface. A ceruloplasmin binding protein with a molecular weight of 60,000 daltons was isolated from human erythrocytes. Erythrocytes which were not protected by ceruloplasmin's antioxidant properties, did not bind ceruloplasmin. Our results provide evidence for the presence of ceruloplasmin receptors in the erythrocyte membrane. It is proposed that the antioxidant activity of ceruloplasmin may play a role in determining the lifespan of circulating red cells.

Ceruloplasmin (Cp) is a multifunctional protein found in the alpha-2-globulin fraction of vertebrate plasma (1). Ceruloplasmin contains a high fraction of the circulating copper in mammalian plasma. Its physiological activities may include the ability to serve as an antioxidant by preventing the accumulation of activated oxygen products which can initiate lipid peroxidation.

Stocks et. al. (2), found that human plasma contained two distinct peaks of antioxidant activity. The main peak was identified as Cp. Ceruloplasmin has also been shown to inhibit Cu(II) catalyzed peroxidation of liposomes (3). Lovstad (4) has shown that human Cp can protect rat erythrocytes from Cu(II) induced lysis, while the addition of catalase or superoxide dismutase had no effect. This was confirmed, independently, by us (5, 6) for a variety of mammalian erythrocytes. We report further studies on the mechanism of Cp's antioxidant properties and on the specific

Abbreviations: Cp, ceruloplasmin; b, bovine; h, human; t, tadpole; RBC, red blood cells.

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interactions between Cp and the red cell membrane. Our data provide evidence for the existence of Cp receptors in the erythrocyte membrane.

Materials and Methods: Erythrocytes were prepared as previously described (5). Ceruloplasmin was isolated from human (hCp) and bovine (bCp) sera by a modification of the method of Zgirski et. al. (7). The Cp isolated showed a single band when subjected to native polyacrylamide gel electrophoresis. The absorbance ratio, A_{610}/A_{280}, was 0.045 for hCp and 0.050 for bCp. Ceruloplasmin was iodinated by the procedure described by Hunter and Greenwood (8). The specific activity of the labeled Cp was 10^6 CPM/pmol. Erythrocytes (20-25 million/ml) were incubated with various amounts of labeled Cp (0.01-0.15 pmol) and unlabeled Cp (1.5-150 pmol) for 16 hours at 4C in a total volume of 1.0 ml. At the end of the incubation the cells were centrifuged and an aliquot of the supernatant was withdrawn for the estimation of unbound Cp. The cells were washed 3x with cold phosphate (.02 M) buffered saline, pH 7.4. An aliquot of the cells was withdrawn and counted in a fresh tube so that Cp non-specifically bound to the incubation tube would not be included with the labeled Cp bound to the cells. Scatchard analysis was used to determine the binding affinity and the number of Cp binding sites on the erythrocytes. Hemoglobin free ghosts were prepared by the method of Pfeffer (9). The ghosts were solubilized by the addition of 0.1% Triton X-100, which was included in all buffers. The extract was incubated in a Cp-agarose column for 2 hr at 23C. Twenty bed volumes of 0.050 M phosphate buffer was used to wash the column, followed by five bed volumes of 0.5 M phosphate. The specifically bound Cp binding protein was eluted with 2.2M phosphate buffer, pH 6.5. The eluted fractions were concentrated and run on 4% SDS polyacrylamide gel and silver stained. The inhibition of Cp binding to erythrocytes was calculated from the following formula: [(CPM bound of ^{125}I Cp) - (CPM bound of ^{125}I Cp in the presence of the indicated protein)]/(CPM bound of ^{125}I Cp).

Results: Human and bovine Cp were labeled with ^{125}I and incubated with erythrocytes from a variety of species. Specific binding of Cp to the red cell was determined by the addition of a large amount of cold Cp to the labeled enzyme (Table 1). When 20 µg/ml cold Cp was added, 40% of the labeled Cp binding was inhibited. When similar amounts of superoxide dismutase or a crude globulin fraction were added, the inhibition was much less, 5% and 7% respectively.

TABLE 1

Compounds added	% Inhibition of binding to RBC by labeled bCp
bCp (unlabeled), 20 µg/ml	40
bSOD, 20 µg/ml	5
Crude globulins, 20 µgl/ml	7

Displacement of bound [^{125}I] bCp by proteins. Proteins were added to 1 ml of an erythrocyte suspension and [^{125}I]-bCp solution. The formula used for calculating percent inhibition is described in Materials and Methods section.

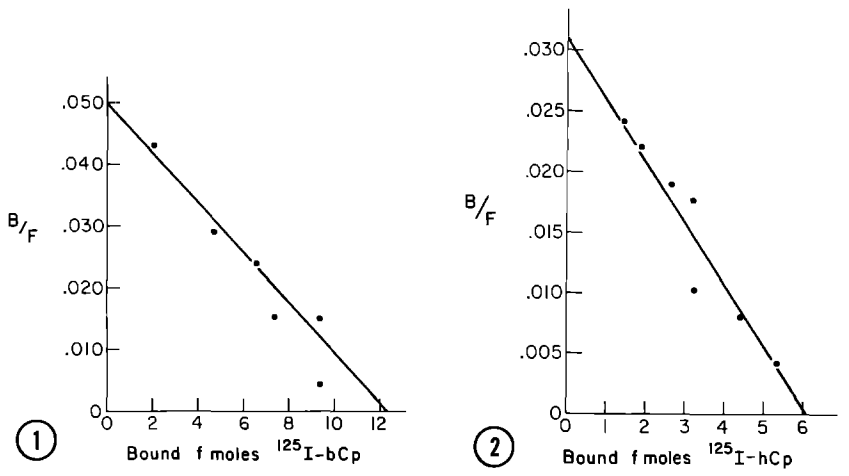

Fig. 1. Scatchard plot of the specific binding of bovine Cp to intact rabbit erythrocytes. Specific binding was determined by subtracting non-specific binding from total binding.

Fig. 2. Scatchard plot of the specific binding of human Cp to intact human erythrocytes. Specific binding was determined by subtracting non-specific binding from total binding.

Fig. (1) shows a Scatchard plot of the binding of hCp by human red cells. A Kd of 5nM was estimated from the slope of this line (r=0.95). The intercept of the x axis was 6.0 fmole indicating 6.0 fmole bound per 25 million cells, corresponding to 144 sites per cell. Fig. 2 is a Scatchard plot of the binding of bCp by rabbit red cells. These data (r=0.96) gave a Kd of 4nM and 294 sites per cell (x intercept of 12.2 fmole per 25 million cells).

A preliminary effort was made to isolate the Cp receptor from human red cell membranes. Hemoglobin free ghosts were prepared from human erythrocytes. The membranes were solubilized with Triton X-100 and incubated with hCp-agarose for two hours at 21C. When the Cp binding protein, purified by affinity chromatography, was subject to SDS-PAGE a single band with a molecular weight of 60,000 was obtained. This band bound to the column again when reincubated with the hCp-agarose column, but the yield was low; about one-fifth of the starting material.

Discussion:

The antioxidant properties of Cp have been extensively studied *in vitro*, but the *in vivo* significance is not understood. Stevens *et. al.* (10) have

reported a Cp binding site in membrane fragments from aortic and heart tissues. We have demonstrated the binding of Cp to an intact cell. The properties of the binding sites and some of its physiological implications are briefly presented here.

Saturation of the specfic binding sites occurs when the difference between the non-specific binding and total binding remains the same as the concentration of ligand is increased. The binding approached saturation when the Cp concentration in the incubation medium approach 1pmol/ml.

Precautions were necessary to minimize non-specific binding. Cell free controls were incubated with labeled Cp to correct for binding to the tube walls. Cells incubated with labeled Cp were transferred to fresh tubes for counting bound Cp. This also prevented the addition of Cp-test tube binding to the total binding. Unbound Cp was determined by the actual free concentration in the extracellular medium, because of the large amount of Cp which binds to the test tube.

Despite all the precautions taken the level of nonspecific binding was still high. This is a peculiarity associated with Cp and is related to phenomena reported earlier (1, 10). At high protein concentrations and low salt, Cp tends to aggregate. Concentrations of Cp used in the binding assay were kept as low as possible, about 1.0 pmol/ml.

When excess cold Cp was added to an equilibrated system of labeled Cp and erythrocytes, 40% of the labeled Cp was displaced. This agrees with the value of 36% displacement reported by Stevens et. al. (10) with chicken Cp and aorta membranes. SOD and beta globulin were much less effective, displacing labeled Cp by only 5 and 7% respectively. This suggests that Cp binds specifically to one or more sites on the erythrocyte.

Red cells which were exposed to trypsin do not bind Cp thereafter. This indicates that the binding site is protein in nature and is probably on the cell surface.

Using Scatchard analysis a Kd of 5nM for the hCp binding site on the human erythrocyte was obtained. The linear plot obtained also suggested a

TABLE 2

REACTANTS	CPM bound to RBC
^{125}I bCp + rRBC	15,535
^{125}I hCp + hRBC	7,781
^{125}I bCp + tRBC	71

The binding of [^{125}I]-Cp by different erythrocytes. [^{125}I]-labeled bCp was incubated with rabbit erythrocytes (rRBC) and tadpole erythrocytes (tRBC). [^{125}I]-hCp was incubated with human erythrocytes (hRBC) for 16 hr at 4C. CPM bound represents the counts per min of labeled Cp bound by the respective erythrocytes.

single class of Cp binding sites. These values indicate a stronger binding than the Kd of 10-50nM reported by Stevens et. al. (10) using membrane fragments from chick aorta and heart. The strength of the binding of Cp to red cells supports the likelihood of a physiological function for the erythrocyte receptors.

If binding of Cp is related to its antilytic properties, then cells which do not bind Cp might not be protected by the addition of Cp. Tadpole erythrocytes were very sensitive to metal ion induced lysis (6). However, when Cp was added to the cell medium lysis was unaffected for all metal ions tried. As can be seen in Table 2 tadpole erythrocytes bind almost no labeled Cp. Ceruloplasmin from different mammalian species crossreact with other mammalian erythrocytes, i.e., bovine Cp protects rabbit erythrocytes, suggesting that binding is required for protection.

Lipid peroxidation has been cited as a prime contributor to erythrocyte death (9). Lipid peroxidation in the red cell membrane can be inhibited by Cp concentrations as low as 10^{-7}M (5). Thus the antioxidant activity of Cp reported in vitro, may play a role in determining the lifespan of circulating red cells in vivo. The relatively small number of binding sites might be a reflection of the efficiency of the antioxidant activity of Cp and its enhancement by the presence of six copper ions in each molecule of Cp.

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References

1. Holmberg, C. G. (1944) Acta Physiol. Scand. 8, 227-229.
2. Stocks, J., Gutteridge, J. M. C., Sharp, J. R., and Dormandy, T. L. (1974) Clin. Sci. Mol. Med. 47, 223-233.
3. Gutteridge, J. M. C. (1983) FEBS LETT. 157, 37-40.
4. Lovstad, R. A. (1982) Int. J. Biochem. 14, 585-589.
5. Barnes, G., and Frieden, E. (1983) Biochem. Biophys. Res. Comm. 115 680-684.
6. Barnes, G., and Frieden, E. (1983) unpublished data.
7. Zgirski, A., Witwicki, J., Helewicz, M., and Witas, H. (1978) Acta Biochem. Pol. 25, 361-368.
8. Hunter, W. M. and Greenwood, F. C. (1972) Nature 194, 495-498.
9. Pfeffer, S. R. and Swislocki, N. I. (1982) Mech. of Ag. and Dev. 18, 355-367.
10. Stevens, M. D., DiSilvestro, R. A. and Harris, E. D. (1984) Biochemistry 23, 261-266.