Binding of Cupric Ions to Bovine Serum Albumin

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Abstract □ The binding of cupric ions to bovine serum albumin was investigated by using a cupric-ion-specific electrode. When using a modified form of the Scatchard equation, it was determined that there are at least two classes of binding sites on bovine serum albumin for cupric ions. One class has three binding sites of relatively strong affinity, with an average binding constant of 3.0×10^6. The other class has about 16 binding sites of relatively weak affinity, with an average binding constant of 2.0×10^4.

Keyphrases □ Cupric ions—binding to bovine serum albumin, determination of number and classes of binding sites, specific ion electrode □ Copper–protein binding interactions—studied using specific ion electrode □ Serum protein binding—binding of cupric ions to bovine serum albumin, studied using specific ion electrode

In the normal human body, the blood serum contains about 114 μg of copper/100 ml of serum. About 94% of this copper is tightly bound to the serum copper–protein ceruloplasmin, an α-globulin with a molecular weight of approximately 151,000. The function of ceruloplasmin is unknown, but it does possess polyphenol oxidase activity (1, 2); it may function in the oxidation of serotonin (3) and epinephrine (4). The remaining 6% of serum copper is loosely bound to serum albumin. The red blood cells also contain about 115 μg/100 ml of packed red blood cells. It has been suggested that there are at least two different fractions of copper in erythrocytes (5), one of which is labile and can be exchanged easily between plasma and red blood cells. The other fraction is present as a copper–protein called erythrocuprein, which contains about 0.34% copper, by weight, tightly bound to the protein.

A hereditary deficiency or absence of ceruloplasmin (6–8) often results in Wilson's disease (hepatolenticular degeneration). This disease results from inordinately high concentrations of copper in many organs and tissues due to a lack of control in absorption of dietary copper or excretion of copper that already has been absorbed or both.

Even though the amount of copper present in the different parts of the blood is known, the individual binding of copper to each component is not well understood. Klotz and Curme (9) studied the thermodynamics of the copper–bovine serum albumin interaction at pH 4.83 and reported that there are 16 binding sites. They had also determined $\Delta F°$ values for the binding of individual Cu^{+2} ions to the protein. However, the analytical method they used to calculate the Cu^{+2} ions in the copper–protein system was sensitive only to about 1×10^{-4} *M* of Cu^{+2} ions. Therefore, it was desirable to reinvestigate the binding of Cu^{+2} to bovine serum albumin with a more sensitive analytical method for the determination of free Cu^{+2} ions. In this regard, the copper-ion-specific electrode seemed very attractive because of its ease of operation. This report presents the use of the specific ion electrode to study the copper–protein binding interaction.

EXPERIMENTAL

Chemicals—The protein solution was prepared by dissolving crystalline bovine serum albumin[1] in distilled deionized water. A 0.100 *M* copper nitrate standard solution[2] was used to prepare the cupric-ion stock solution.

Apparatus—A cupric-ion-specific electrode[3], a single-junction reference electrode[4], and a digital pH/mv meter[5] were used for measurement of the electric potential from which the cupric-ion concentrations were calculated.

Procedure—Fifty milliliters of 5.0×10^{-6} *M* bovine serum albumin (molecular weight assumed to be 69,000), prepared in deionized distilled water, with ϵ at 280 nm equal to 4.60×10^4 liters $mole^{-1}$ cm^{-1} (10), was placed in a 100-ml beaker. The reference electrode, the cupric-ion electrode, and a thermometer were dipped into this solution, which was stirred magnetically. The temperature was maintained at 25.0° using a constant-temperature bath.

Copper nitrate solution, 1.0×10^{-2} *M*, was added to the bovine serum albumin in 10-μl increments with a micropipet[6]. Both the bovine serum albumin and the copper nitrate solutions were adjusted to pH 5.2 with trace amounts of sodium hydroxide or sulfuric acid, as appropriate. For samples below 10^{-3} *M* in total ionic strength, the difference in total ionic strength between samples and standards may be as large as a factor of 5 without resulting in serious errors (11). The 5.0×10^{-6} *M* protein and 1.0×10^{-2} *M*

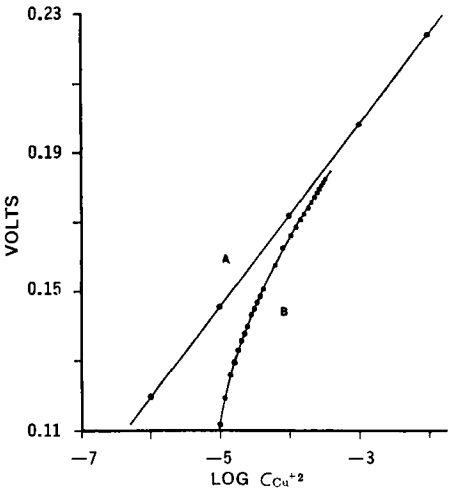

Figure 1—*Specific ion electrode response, in volts, as a function of formal cupric-ion concentration in the absence of bovine serum albumin (A) and in the presence of 5.0 × 10⁻⁶ M bovine serum albumin (B).*

[1] Calbiochem, San Diego, Calif.
[2] Orion Research Inc., Cambridge, Mass.
[3] Model 94-29, Orion Research Inc., Cambridge, Mass.
[4] Model 90-01, Orion Research Inc., Cambridge, Mass.
[5] Model 801, Orion Research Inc., Cambridge, Mass.
[6] Centaur Chemical Co., Stamford, Conn.

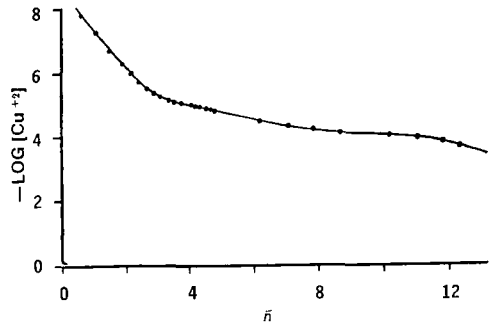

Figure 2—*Plot of* $-log\ [Cu^{+2}]$ versus $\bar{n}$ *for the bovine serum albumin–*Cu^{+2} *system at pH 5.2.*

copper nitrate solutions in distilled deionized water had a pH around 5, so only trace amounts of sulfuric acid or sodium hydroxide were needed to adjust the pH to 5.2. Such small quantities of acid or base are not expected to change the ionic strength enough to affect the measurement of the cupric-ion concentration. Ideally, the binding study should be carried out at the biological pH, which is 7.4; but at a pH of 6 or higher, some Cu^{+2} is hydrolyzed to hydroxo complexes, which may or may not precipitate. Hence, pH 5.2 was chosen for the present study.

The instrument was standardized before and after each titration, using various exact concentrations of pure copper nitrate solutions. By knowing the total amount of cupric ion added and the concentration of free cupric ions, the amount of copper bound was calculated.

RESULTS AND DISCUSSION

The potentiometric data for the titration of bovine serum albumin with copper nitrate are presented graphically in Fig. 1. Curve A is the standard curve for the known concentrations of cupric ions in the absence of the protein. Curve B represents the electromotive force in volts during the bovine serum albumin–copper nitrate titration as a function of total Cu^{+2} added.

The binding of cupric ions to bovine serum albumin, *P*, can be represented by a series of equilibria (Scheme I):

$$P + Cu^{+2} \overset{k_1}{\rightleftharpoons} P(Cu^{+2})$$
$$P(Cu^{+2}) + Cu^{+2} \overset{k_2}{\rightleftharpoons} P(Cu^{+2})_2$$
$$\vdots \qquad\qquad \vdots \qquad\qquad \vdots$$
$$P(Cu^{+2})_{i-1} + Cu^{+2} \overset{k_i}{\rightleftharpoons} P(Cu^{+2})_i$$

Scheme I

where $P(Cu^{+2})_{i-1}$ and $P(Cu^{+2})_i$ represent protein–copper complexes with $i-1$ and i cupric ions, respectively. The number i may vary from 1 to N, the maximum number of cupric ions that can bind to the protein. The k's are the respective equilibrium constants corresponding to each equilibrium.

The number of cupric ions bound per mole of bovine serum albumin, $\bar{n}$, at any point in the titration is related to the N equilibrium constants by the expression (12):

$$\bar{n} = \frac{\sum_1^N i\left(\prod_1^i k_i\right)[Cu^{+2}]^i}{1 + \sum_1^N \left(\prod_1^i k_i\right)[Cu^{+2}]^i} \qquad \text{(Eq. 1)}$$

where $[Cu^{+2}]$ indicates the equilibrium concentration of free cupric ions, and k_i is the equilibrium constant for the binding of ith cupric ion. If the individual equilibrium constants differ widely from each other (by at least two orders of magnitude) such that only one equilibrium is experimentally detectable at any point in the titration, Eq. 1 predicts that a plot of $-\log [Cu^{+2}]$ *versus* $\bar{n}$ should show N distinct inflection regions. The midpoint of these inflections should correspond to the respective equilibrium constants. Moreover, it also predicts that as $\bar{n}$ approaches N, $-\log [Cu^{+2}]$ should decrease sharply.

Figure 2 shows a plot of $-\log [Cu^{+2}]$ *versus* $\bar{n}$ for the bovine serum albumin–Cu^{+2} system. There are no clear inflection regions, a result that indicates strongly overlapping successive equilibria. The fact that a definitive saturation plateau has not been reached even at $\bar{n} = 12$ indicates that there are more than 12 binding sites. Therefore, the binding data should be analytically fitted to Eq. 1 (which will have $N + 1$ unknowns) to obtain precise binding parameters. However, if the N binding sites for cupric ions on bovine serum albumin are electrostatically noninteracting, and if the protein has identical affinity for each of the N cupric ions (*i.e.*, $k_1 = k_2 = k_3 \ldots = k_N$), then the relationship of $\bar{n}$ to the overall binding constant, K, becomes simpler and can be expressed by the Scatchard equation (13):

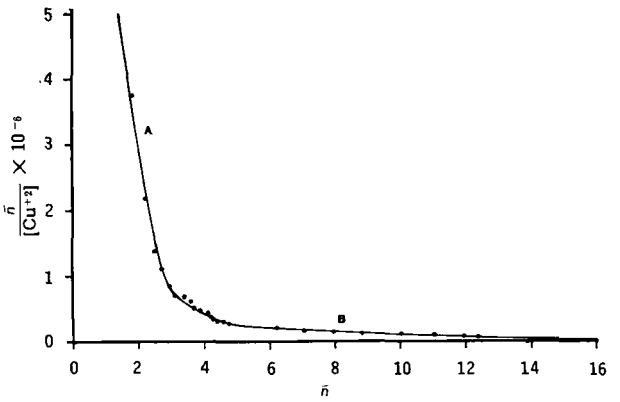

Figure 3—*Scatchard plot for the binding of* Cu^{+2} *ions to bovine serum albumin.*

$$\bar{n} = Nk[Cu^{+2}]/(1 + k[Cu^{+2}]) \qquad \text{(Eq. 2)}$$

which, on rearrangement, gives:

$$\bar{n}/[Cu^{+2}] = Nk - \bar{n}k \qquad \text{(Eq. 3)}$$

According to Eq. 3, a plot of $\bar{n}/[Cu^{+2}]$ *versus* $\bar{n}$ (known as a Scatchard plot) should be a straight line with the slope $= -k$ and the X intercept $= N$. Figure 3 shows the Scatchard plot for the bovine serum albumin–Cu^{+2} system. Clearly, these data do not fit Eq. 3. Klotz and Curme (9) also found that their data for the bovine serum albumin–Cu^{+2} system did not fit this linear expression. However, if it is assumed that there are two classes of binding sites that are widely different from each other in their binding affinities, the Scatchard Eq. 2 can be modified to:

$$\bar{n} = \{N_1k_1[Cu^{+2}]/(1 + k_1[Cu^{+2}])\} + \{N_2k_2[Cu^{+2}]/(1 + k_2[Cu^{+2}])\} \qquad \text{(Eq. 4)}$$

where N_1 is the number of binding sites of the first kind with a binding constant of k_1, and N_2 is the number of binding sites of the second kind with a binding constant of k_2. In this case, the ideal Scatchard plot, instead of being a straight line, is a hyperbola composed of two straight-line portions.

The binding data of the bovine serum albumin–Cu^{+2} system do yield a hyperbola made up of two straight-line portions (Fig. 3), indicating that there are at least two classes of binding sites on bovine serum albumin for cupric ions. The straight-line segment, A, of Fig. 3, gives an X intercept of about 3, indicating one class of binding has three sites. The slope of this line, which is equal to the average binding constant, is 3×10^6. The second straight-line segment, B, gives an X intercept of about 16, revealing that the second class of binding has about 16 sites. From the slope of segment B, the average binding constant for the latter sites is 2×10^4.

The number of binding sites for the second class of binding is the same as that reported by Klotz and Curme (9). However, they calculated the change in free energy of the individual 16 equilibria, the first of which had a k value of 1.8×10^4 ($\Delta F^\circ = -5908$ cal/mole at 25°). This value agrees with the value obtained from the Scatchard plot.

Thus, it appears that cupric ions bind to bovine serum albumin through at least two different classes of binding sites; one accom-

modates only about three cupric ions, which are relatively tightly bound, while the other accommodates about 16 cupric ions, which are rather weakly bound. It is possible that the first three cupric ions bind intimately by penetrating the protein while the rest of the cupric ions bind weakly to the surface of the protein.

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Synthesis of 2-(4-Arylthiosemicarbazidocarbonylthio)benzthiazoles and Their Monoamine Oxidase Inhibitory and Anticonvulsant Properties

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Abstract □ Ten 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles were synthesized, characterized, and evaluated for their monoamine oxidase inhibitory and anticonvulsant activities. All substituted benzthiazoles inhibited activity of monoamine oxidase in rat brain homogenate where the degree of enzyme inhibition was higher with kynuramine as compared to tyramine and 5-hydroxytryptamine as the substrates. All substituted benzthiazoles possessed measurable anticonvulsant activity against pentylenetetrazol-induced convulsions.

Keyphrases □ 2-(4-Arylthiosemicarbazidocarbonylthio)benzthiazoles—synthesis, characterization, monoamine oxidase inhibitory and anticonvulsant activities □ Monoamine oxidase inhibitory activity—substituted benzthiazoles, relationship between enzyme inhibitory and anticonvulsant activities, rat brain homogenate □ Anticonvulsant activity—substituted benzthiazoles, relationship to monoamine oxidase inhibitory activity, mice

Benzthiazoles have been reported to possess diverse pharmacological properties (1, 2). Recent studies indicated anticonvulsant and monoamine oxidase [EC 1.4.3.4 monoamine: O_2 oxidoreductase (deaminating)] inhibitory properties of substituted benzthiazoles (3). In addition, the ability of hydrazines (4), semicarbazides (5, 6), and thiosemicarbazides (7) to inhibit monoamine oxidase and the anticonvulsant activities possessed by the inhibitors of monoamine oxidase (8) led to the synthesis of 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles. In the present study, all substituted benzthiazoles possessed anticonvulsant activity and inhibited monoamine oxidase activity of rat brain homogenate.

The various substituted benzthiazoles were synthesized by following the methods outlined in Scheme I.

EXPERIMENTAL[1]

Chemistry—Ten 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles were synthesized from 2-mercaptobenzthiazole. 2-Mercaptobenzthiazole was first treated with ethyl chloroformate in the presence of dry acetone and anhydrous potassium carbonate to yield 2-(ethoxycarbonylthio)benzthiazole, and then the resulting product was converted into 2-(hydrazinocarbonylthio)benzthiazole by refluxing with hydrazine hydrate on a steam bath in absolute ethanol. The hydrazide thus formed was refluxed with suitable arylisothiocyanates in dry benzene to form the desired 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles (Compounds I–X).

2-(Ethoxycarbonylthio)benzthiazole—A mixture of 2-mercaptobenzthiazole (0.01 mole) in dry acetone, containing anhydrous potassium carbonate (0.011 mole) and ethyl chloroformate (0.01 mole), was refluxed on a steam bath for 16–18 hr. The mixture was filtered and the excess acetone from the filtrate was removed by distillation. The solid mass, which separated on cooling, was collected by filtration, washed with water, and recrystallized from ethanol, mp 63° [lit. (9) mp 64°].

[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes with partial immersion thermometer and are corrected.