Intrinsic stoichiometric equilibrium constants were determined for zinc(II) and copper(II) binding to bovine and human serum albumin. Data were obtained from equilibrium dialysis experiments. Metals were presented to apoprotein as metal chelates in order to avoid metal hydrolysis and to minimize nonspecific metal-protein interactions. Scatchard analysis of the binding data indicated that the high affinity class for both zinc and copper was comprised of one site. Results of binding experiments done at several pH values suggested that while both histidyl and carboxyl groups appear to be involved in copper binding, histidyl residues alone were sufficient for zinc binding. These amino acid residues were used in combination to model several binding sites used in the formulation of equilibrium expressions from which stoichiometric constants were calculated. The log$_{10}$K for bovine serum albumin were calculated to be 7.28 for Zn(II) and 11.12 for Cu(II). Those for human serum albumin were determined to be 7.53 and 11.18 for Zn(II) and Cu(II), respectively. These constants were used in equilibria to simulate speciation of metal-albumin and metal-chelator and to illustrate relative binding affinities. This comparison of binding strengths was possible only through the calculation of an intrinsic stoichiometric binding constant.

In order to understand fully the structure and function of metalloproteins, it is vital to characterize their binding of di- and trivalent metal ions. Metal specificity, number of binding sites, intrinsic binding constants, and the chemical nature of the ligands at the sites are parameters of major consequence. Serum proteins, particularly serum albumin, play an essential role in the transport and metabolism of Zn(II) and Cu(II). Consequently, the interactions of these metals with serum albumin have been extensively studied. Although an abundance of physicochemical data regarding serum albumin is available, there is still disagreement about the number and affinity of Zn(II) and Cu(II) sites (Table I). The literature reveals a chronological trend toward a lower number of binding sites, $n$, due to a shift in modeling from many, simple sites (e.g. one metal atom per surface residue) to fewer, more complex sites. However, even when identical $n$ values are reported, the $K$ values may vary by orders of magnitude.

Some of the ambiguity about Zn(II) and Cu(II) binding is a result of the ligands at the sites and their affinities. This comparison of binding strengths was possible only through the calculation of an intrinsic stoichiometric binding constant.

In order to determine the number of sites, metal binding can be measured using a variety of techniques. Complex curve-fitting routines of such data have been devised to differentiate classes of binding directly from these profiles (Klotz and Hunston, 1971; Feldman, 1972; Blondeau and Robel, 1976; Kamikubo et al., 1986). The binding constants derived from these routines are apparent constants and therefore give little information about the nature of the binding ligand or its affinity relative to other ligands. The most meaningful comparison of binding activity requires knowing the intrinsic binding constant, analogous to the stability constant for low molecular weight chelates. For example, the stability constant for Zn(II) and histidine is given by:

\[ \text{Zn}^{2+} + \text{His}^- \rightleftharpoons \text{ZnHis}^+ \]  

\[ K = \frac{[\text{ZnHis}^+]}{[\text{Zn}^{2+}][\text{His}^-]} \]  

In order to determine this type of constant, three values must be known: the concentration of complex, the concentration of ionic metal, and the concentration of totally deprotonated ligand.

We present here a more definitive analysis of the metal-albumin system. We define a self-consistent system in which intrinsic binding constants are determined for the binding of Zn(II) and Cu(II) to BSA and HSA. These constants can then be used to describe effectively the metal binding activity of albumin in the complex milieu of serum.

---

*This work was supported in part by United States Public Health Service Grant GM 07539 and by National Institutes of Health Grant DK 12386-25. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked 'advertisement' in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

1 The abbreviations used are: BSA, bovine serum albumin; HSA, human serum albumin; Mes, 2-(N-morpholino)ethanesulfonic acid; Hepes, N-2-hydroxyethylpiperezine-N'-3-propanesulfonic acid; $V$, [molar bound metal]/[molar total protein].
MATERIALS AND METHODS

Albumin Preparation—Stock solutions of BSA (Sigma, M, = 66,000, 7.92 mg/ml) and HSA (Sigma, M, = 66,000, 6.58 mg/ml) were made by dissolving albumin in buffer (250 mM NaCl, 30 mM Hepes, pH 7.0) and dialyzing exhaustively against buffer at 4 °C. The solutions were filtered through a 0.45-μm nylon membrane, and aliquots were stored at -20 °C until use. Protein concentration was determined by the method of Lowry et al. (1951). Purity of these stock solutions was shown by SDS-polyacrylamide gel electrophoresis. Levels of Zn(I1) and Cu(I1) following dialysis were measured by furnace atomic absorption spectros- 

SDS-polyacrylamide gel electrophoresis. Levels of Zn(I1) and Cu(I1) following dialysis were measured by furnace atomic absorption spectroscopy (Hitachi Model 180-80, Hitachi, Ltd., Tokyo). 

Radioisotope Solutions—[65Zn]ClO4 (Du Pont-New England Nuclear; specific activity 4.25 mCi/mg) and [65Cu]ClO4 (Los Alamos National Laboratory, Los Alamos, NM) were diluted in 0.1 N HCl prior to use. [65Zn]ClO4 measurements were made in a Tracor Analytic GammaTrac Model 1191 y counter set to capture the 1.1-1.2 MeV emission. Five microliters of [65Cu]ClO4 diluted stock were added to 5 ml of scintillation mixture, and aliquots were measured in a Beckman Model LS-233 liquid scintillation counter using a window which captures the 0.7-1.71 MeV emissions. The scintillation mixture contained toluene, Omnifluor (Du Pont-New England Nuclear; 4 g/l), glacial acetic acid (25 ml/liter), and Triton X-100 (250 g/liter). Aliquots (5 μl) of the copper stock were periodically counted during the short lifetime of the isotope to ensure that 67Cu activity remained at least twice background.

Chelate Solutions—All metals ([ZnSO4]7H2O, CaSO4, 5H2O), buffers (Hepes, Mes, Hepps), salt (NaCl), and chelators (glycine, histidine) used in these experiments were of the best commercial grade. Hepes buffer was used for the experiments conducted at pH 7.0, Hepps for those done at pH 8.5, and Mes for those done at pH 5.0. Water obtained by repeated sub-boiling distillation in quartz was used throughout to reduce trace element contamination. Chelate solutions were prepared by mixing the components in the following order: 1) the radioisotope and the corresponding metal salt at unadjusted pH (pH < 5.0); 2) chelator; 3) buffer and NaCl to a final concentration of 30 mM and 250 mM, respectively; 4) small amounts of 0.1 N NaOH to bring the solution to the desired pH; and water to volume. The metal-to-chelator ratio was 1:2 throughout. [65Zn]ClO4 represented no more than 5% of the total zinc. [65Cu]ClO4 was present only as a tracer, and the amount added had no significant effect on the final concentration of metal.

Equilibrium Dialysis—Experiments were performed in acrylic chambers coated with a thin film of anion exchange material and separated by a single thickness of cellulose dialysis tubing (Spectra/Por, Spectrum Medical Industries, Inc., Los Angeles, CA) as described by Malmstrom (1953). One ml of a metal-chelate solution (diffuse) and 1 ml of a protein solution (retentate) were placed opposite sides of the membrane. For a given experiment, protein concentration was held constant, and the concentration of metal-chelate was varied over a range of metal:protein ratios from 0.5:1 to 80:1. The solutions were agitated on a reciprocal shaker (approximately 1 cycle/sec) and allowed to reach equilibrium at 4 °C for 3 days. Samples (900 μl) were withdrawn from each cell compartment for measurement of final pH and radioactivity.

Metal Speciation—For each chamber, the fraction of metal bound to protein was calculated by the following relationship:

\[
\text{Fraction bound} = \frac{\text{cpm}_{\text{bound}} - \text{cpm}_{\text{free}}}{\text{cpm}_{\text{bound}} + \text{cpm}_{\text{free}}} \quad \text{(Eq. 3)}
\]

The concentration of metal bound to protein and the concentration of free metal were calculated using:

\[
[M]_{\text{bound}} = [M]_{\text{total}} \times \text{fraction bound} \quad \text{(Eq. 4)}
\]

and

\[
[M]_{\text{free}} = [M]_{\text{total}} - [M]_{\text{bound}} \quad \text{(Eq. 5)}
\]

respectively. Free metal is thus defined as any metal not bound to the protein at equilibrium.

The concentration of total ligand, concentration of free metal, and pH were used as input for equilibria equations to determine chelate speciation. The stoichiometric equations and binding constants for the Zn(I1)-histidine equilibria were those of Petit and Swash (1976). Those for the Cu(I1)-histidine equilibria were from Kruck and Sarkan (1973). The Zn(I1)- and Cu(I1)-glycine equilibria and constants were taken from Martell and Smith (1974). These equilibria expressions were used to calculate the concentration of ionic metal at equilibrium for each metal-chelator pair (Hegnauer et al., 1979). The concentration of ionic metal in the metal-chelator-protein system was set equal to that calculated for the metal-chelator system.

RESULTS

Binding Profiles—Zn(I1) and Cu(I1) were presented to BSA as the histidine chelate. Typical binding profiles for these experiments are shown as Scatchard plots (Scatchard, 1949; Figs 1 and 2). Results of preliminary graphical analysis suggested the presence of two types of binding (Klotz and Hunston, 1971).

Apparent constants calculated via this analysis were used to estimate the contribution of each component to the overall concentration of bound metal. Binding due to a given component was visualized by plotting V versus log[M]free. Saturation is indicated by sigmoidicity and by attaining a plateau at high metal concentrations. As shown in Fig. 3, the first type of binding is saturated over the experimental range. The second component observed did not reach a plateau on this type of plot and cannot be considered saturable over the experimental range of metal concentration (data not shown).

Number of Binding Sites—The point of saturation observed correlates well with the break in the data evident in the Scatchard plots. This evidence was used as the basis for dividing the data into two linear groups. Linear regression, in which error in x and y are given equal weight, was performed on each set of points (Reilman et al., 1986). The value of V at which these lines intersected was taken as the best statistical representation of n (Dyke et al., 1987).

Table I

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Method*</th>
<th>pH</th>
<th>n*</th>
<th>Log10 K*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanford (1952)</td>
<td>1952</td>
<td>P</td>
<td>5.82</td>
<td>17</td>
<td>2.9</td>
</tr>
<tr>
<td>Saroff and Mark (1953)</td>
<td>1953</td>
<td>P</td>
<td>6.1</td>
<td>8</td>
<td>3.1</td>
</tr>
<tr>
<td>Rao and Lal (1958a)</td>
<td>1958</td>
<td>P</td>
<td>6.5</td>
<td>2</td>
<td>3.9</td>
</tr>
<tr>
<td>Osterberg (1971)</td>
<td>1971</td>
<td>PT</td>
<td>6.78</td>
<td>1</td>
<td>9.6</td>
</tr>
<tr>
<td>Rall (1974)</td>
<td>1974</td>
<td>ED</td>
<td>7.4</td>
<td>1</td>
<td>7.8</td>
</tr>
<tr>
<td>Giroux and Schoun (1981)</td>
<td>1981</td>
<td>U</td>
<td>7.5</td>
<td>1</td>
<td>7.9</td>
</tr>
<tr>
<td>Copper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klotz and Curme (1948)</td>
<td>1948</td>
<td>ED</td>
<td>4.83</td>
<td>16</td>
<td>4.3</td>
</tr>
<tr>
<td>Tanford (1952)</td>
<td>1952</td>
<td>P</td>
<td>4.96</td>
<td>17</td>
<td>2.7</td>
</tr>
<tr>
<td>Rao and Lal (1958b)</td>
<td>1958</td>
<td>ED</td>
<td>6.5</td>
<td>16</td>
<td>4.88</td>
</tr>
<tr>
<td>Giroux and Schoun (1981)</td>
<td>1981</td>
<td>U</td>
<td>7.5</td>
<td>1</td>
<td>13.2</td>
</tr>
</tbody>
</table>

* P, polarography; ED, equilibrium dialysis; PT, potentiometry; U, ultrafiltration.

n, number of binding sites.

log10 of the reported apparent binding constant.

Protein and metal were combined at a 1:1 ratio prior to equilibrium.

Calculated for first copper bound. Table 3, pg. 841.
Affinity of Serum Albumin for Zn(II) and Cu(II)

Metal-Protein Equilibria—As stated above, our desire was to determine the intrinsic binding constant for BSA. The expression for such a constant would be:

$$nM^{z+} + P^z = M_jP^{-2z}$$  \hspace{1cm} (Eq. 6)

$$K = \frac{[M_jP^{-2z}]}{[M^{z+}][P^z]}$$  \hspace{1cm} (Eq. 7)

However, an intrinsic constant requires the concentration of totally deprotonated ligand. The determination of the protonation state of an entire protein molecule is problematic. We have solved this problem by switching from a metal-protein to a metal-site scheme. That is:

$$M^{z+} + B^{-} = MB^{-z+2}$$  \hspace{1cm} (Eq. 8)

$$K = \frac{[MB^{-z+2}]}{[M^{z+}][B^{-}]}$$  \hspace{1cm} (Eq. 9)

where $[MB^{-z+2}]$ is the concentration of bound metal and $B^{-x}$ is an empty, deprotonated binding site. The concentration of total binding sites is calculated by: $[B]_T = n[P]_T$, where $[P]_T$ is the total concentration of protein and $n$ is the number of sites from Scatchard analysis.

Protonation State of the Ligand—$[B]_T$ can also be expressed as:

$$[B]_T = [MB^{-z+2}] + [B^{-z}] + [HB^{-z+2}] + \ldots + [H,B]$$  \hspace{1cm} (Eq. 10)

where the first term is determined by experimental data and the remainder of terms describe the protonation state of the binding site.

Although the exact makeup of a given binding site cannot be known a priori, it can be effectively modeled. Equilibrium dialysis experiments were performed at several pH values (5.0, 7.0, and 8.5) in order to vary the protonation state of albumin. Binding data were plotted as Scatchard graphs and compared. The results for Zn(II) binding at pH 7.0 are shown in Fig. 1, with $n = 1$ as described in the previous section. The binding profile at pH 8.5 showed no change relative to the pH 7.0 binding profile. Experiments at pH 5.0, however, indicated that no apparent binding occurred ($V = 0$, data not shown). In the case of Cu(II), the binding profiles at all three pH values were identical. These results suggest that histidine alone is sufficient to bind zinc. They also suggest that while histidine is a likely candidate for copper binding, carboxyl residues may be involved.

Calculation of Binding Constants—Results from equilibrium dialysis experiments were used to calculate the stoichiometric binding constant for each metal. The potential ligands of the binding site were used in various combinations to produce several models. The intrinsic protonation constants for the dissociable groups on BSA determined by Tanford et al. (1955) were used to describe the protonation state of the ligands in each model site. The concentration of ionic metal in the metal-chelator-protein site system at equilibrium was determined as stated under "Materials and Methods." These values were placed into the metal-protein equilibrium expression to calculate an intrinsic binding constant for various model binding sites. The model which resulted in the lowest coefficient of variation was chosen as the best statistical representation of the binding constant. The log$_{10}$K for Zn(II) binding to BSA was determined to be 7.28, and that for Cu(II) binding, 11.12 (Table II).

Binding to HSA—Another serum albumin, HSA, was used as a second subject for the analysis system. As expected, the results for HSA, in terms of $n$ and suggested binding ligands, are available upon request.
were identical with those for BSA. The $\log_{10} K$ values for Zn(II) and Cu(II) binding to HSA are 7.53 and 11.18, respectively (Table II).

**DISCUSSION**

The present work defines the conditions necessary for the determination of intrinsic constants which describe the binding of metals to proteins. The most important consideration is the presentation of metal in the form of a well-characterized chelate. This method has three distinct advantages over those used in the past. First, chelation prevents polymerization of metal ions at nonacidic pH. By occupying metal coordination sites, the chelator prevents the hydroxide binding which leads to polymer formation. Second, the concentration of metal species in solution can be calculated. In the absence of a chelator, these concentrations cannot be known with any confidence. Third, the chelator acts as a buffer for unbound metal ions. For a given concentration of metal and chelator and for a given pH, the concentration of unbound metal will remain constant once equilibrium is reached. Only an entity of higher affinity (e.g., a protein binding site) will bind this “free” metal while other, more nonspecific, interactions between metal and protein will be minimized.

Given that the proper chelator is used, only the highest affinity class will be observed to bind metal. Experimental data would then be expected to produce a Scatchard plot which is linear until the sites in the highest affinity class are saturated. Under ideal circumstances, binding would no longer be observed once these sites are saturated and $V$ would go to 0. In reality, binding is visualized as in Figs. 1 and 2 with a more horizontal tailing portion at high $V$ values. The current method of interpreting these profiles involves the use of multiple binding classes, multiple $n$ values, and multiple binding constants as parameters for fitting the data set. This curve-fitting, by the use of excessive parameters, may introduce artificiality into the system for the sake of fit.

Interpretation of these graphs can be simplified if some background information is introduced. For example, when a well-characterized chelate is used to present metal to protein, the relative strength of the chelator is known. Use of a chelator which is stronger than any protein binding site will result in no observed binding. Use of a relatively weak chelator may result in observed binding at sites other than those in the highest affinity class. It is therefore possible to identify empirically a chelator which permits binding at the lowest affinity class while minimizing interaction at any other class of lower affinity sites.

The optimum situation would be one in which no interactions occur at these lower affinity sites. Experimentally, this is not the case. Metal binding is measured at equilibrium, a condition under which all metal species exist. As long as ionic metal is present, it is possible for this species to interact with the protein surface in a nonspecific manner. The probability of this occurring will increase with increasing total metal-chelate concentration. Any interaction with the low affinity sites will be reflected in the data as metal binding. Such binding is unsatetable in the experimental range and proportional to $[M]_T$. As a result, $V$ will continue to increase until the protein is "coated" with metal.

Upon saturation of the high affinity site(s), any increase in $V$ will be due to nonspecific interaction alone. Also, any further addition of metal-chelate will behave as, and be reflected in, the equations as unbound metal ($\Delta[M]_I = \Delta[M]_{free}$). Since both the change in $V$ and the change in $[M]_{free}$ are proportional to the change in $[M]_T$, $V/[M]_{free}$ will approximate a constant.

A Scatchard plot would then be expected to have two domains: (i) a steep portion at low $V$ values representing binding at the high affinity site and (ii) a nearly horizontal portion at high $V$ values representing nonspecific interactions at the protein surface. The nonspecific binding beyond saturation can be thought of as a new x-axis, and thus the intersection of the two regression lines becomes the equivalent of the intercept on the x-axis. It is worth noting that, in this case, this intercept is not precisely equal to 1. Although fractional binding sites have been suggested in the case of ferritin subunits, we have no evidence that this is the case for albumin. We have thus taken the results from the Scatchard plots to be the nearest integer value.

Some previous investigators have also determined an $n$ of 1 for Zn(II) and Cu(II) binding to BSA. In their experiments, metal salts were combined, perhaps fortuitously, with apoprotein at a 1:1 ratio under conditions which enable nonspecific binding to sites other than the one of interest. The competition for metal between the protein-metal complex and free chelator was then determined by equilibrium dialysis. A potential criticism of this approach is that the number of binding sites is limited to 1 by experimental design. The reality and validity of the calculated constant will be affected by this limitation. By presenting chelated metal to apoprotein over a saturating range, we have unambiguously demonstrated the presence of one strong site.

The formulation of our equilibria for the calculation of $K$ requires that the protonation state of the binding site be known. The results from the pH studies provided strong evidence for the involvement of histidine in both zinc and copper binding and carboxyl groups in copper binding. This kind of evidence will be useful in narrowing the focus of spectroscopists to characterize more readily the true nature of the binding site. Once this is done, the protonation equilibria of the binding site will no longer need to be modeled and a final value of the intrinsic constant can be calculated. The structure of HSA has recently been presented by He and Carter (1992). Final definition of the HSA binding constants will soon be possible.

The constants determined in this study are intrinsic binding constants which describe the interaction of metal ions with a specific structural binding site, rather than with an entire protein. It is thus possible to use these constants to calculate speciation of a metal-BSA system, as has been done for the metal-His and metal-Gly systems, and to compare relative strengths of binding. This comparison is best illustrated by the concentration of ionic metal at equilibrium in the presence of chelators. A simple numerical comparison of association constants is inappropriate since $[M]^{-2}$ and the concentrations of other species will change as a function of pH (Hegenauer et al., 1979).

Fig. 4 illustrates the type of chelate comparison now possible. A higher pH indicates a lower $[M]^{-2}$ and thus stronger chelation. The relative strength of chelation can then be determined at any given pH. Glycine, a weaker chelator than histidine, would be expected to give up metal to several classes of BSA sites. By using histidine as the chelator, binding at these lower

<table>
<thead>
<tr>
<th>Table II</th>
<th>Affinity of Serum Albumin for Zn(II) and Cu(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal</td>
<td>$n^a$</td>
</tr>
<tr>
<td>Zinc</td>
<td>1</td>
</tr>
<tr>
<td>Copper</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ Number of binding sites in highest affinity class.

$^b$ $\log_{10}$ of the stoichiometric binding constant.

$^c$ Values are presented as mean ± sample S.D. (n).
affinity sites would be minimized to the point of being unobservable. If an even stronger chelator, such as nitrilotriacetic acid, had been used to present metal to BSA, we would expect to observe no binding since even the highest affinity site on BSA cannot successfully compete with this chelator. Results of experiments in our laboratory have borne out this conclusion.4

Our system of analysis to obtain intrinsic binding constants is of immediate relevance to two issues of trace metal metabolism. The metal ion distribution in biofluids, particularly serum, has been of interest for some time. A great deal of effort has gone into the simulation of metal distribution among the various serum components (May et al., 1976; Berthon et al., 1978, Harris and Keen, 1989). Without a stoichiometric binding constant for the serum proteins, their inclusion in the distribution models has been problematic.

Comparisons can now be made between the affinity of Zn(II) and Cu(II) for BSA and other albumins. Dog serum albumin is of particular interest since it has been claimed that this protein lacks a strong affinity site for Cu(II) (Sass-Kortsak et al., 1967). Sequence analysis has shown that the histidine at position 3 is replaced by a tyrosine, which may account for the reported reduced Cu(II) affinity (Dixon and Sarkar, 1972). Similar sequence changes are seen in naturally occurring mutations of human serum albumin (Takahashi et al., 1987). By comparing the relative binding strengths of these proteins to that of BSA, it will be possible to determine if the strongest site class is necessary for effective metal binding and transport and if the absence of the strongest site affects metal metabolism.

We have presented Zn(II) and Cu(II) to albumin in the form of well-characterized chelates, avoiding the problems of metal hydrolysis. As a result, we have been able to model a binding site and to calculate an intrinsic binding constant. Whereas much of the work describing the interaction of these metals with albumin has been done using peptides, our results provide information about the amino acid ligands in the intact protein. It is now possible to include serum albumin in the biofluid models and to predict the precise role of albumin in metal metabolism.

Acknowledgment—We thank David Clopton for valuable criticisms and assistance in programming.

REFERENCES

Hegenauer, J., Saltman, P., and Nace, G. (1979) Biochemistry 18, 3865-3875