Metal binding strategies employing low molecular weight chelators and equilibrium dialysis were used to investigate several unresolved aspects of zinc and copper binding to serum albumin. Direct measurement of histidine binding to bovine serum albumin when the histidine is presented either as a metal-chelate or alone provides no evidence for an albumin-metal-histidine ternary complex. Using previously determined intrinsic constants for Zn(II) and Cu(II), we have measured zinc binding to bovine serum albumin in the presence of saturating amounts of copper. The results of these experiments unambiguously show that zinc and copper bind at separate noninteracting sites on this protein. The intrinsic constants for zinc and copper binding to dog serum albumin have been determined. Contrary to previous reports, we find that dog serum albumin has a specific high affinity site for copper, logK = 10.17 for Cu(II) compared to 6.85 for Zn(II) at the separate site.

The characterization of metal binding to metalloproteins is vital to our understanding of the relationship between structure and function. A fundamental parameter of metal binding activity is the intrinsic binding constant, which describes the affinity with which metal ions are bound to specific protein sites. We have recently developed a self-consistent experimental strategy with which these constants can be rapidly and accurately determined (1).

The central tenet of the strategy is the presentation of the metal to the protein in the form of well characterized, low molecular weight chelates to prevent metal hydrolysis and nonspecific binding. Further, this strategy permits the determination of the number of specific metal binding sites and provides insights regarding the amino acid ligands involved in binding.

In our previous paper, we used this strategy to determine the intrinsic binding constants for Zn(II) and Cu(II) binding to bovine serum albumin (BSA). We have applied this experimental system to other aspects of Zn(II) and Cu(II) binding to BSA. In particular, we tested whether an albumin-metal-chelator ternary complex formed and whether the high affinity sites for Zn(II) and Cu(II) are identical or interactive.

The formation of an albumin-metal-chelator ternary complex has been proposed by previous investigators (2-4). Lau and Sarkar (3), using spectroscopic and equilibrium dialysis methods, reported the presence of an HSA-Cu(II)-Histidine ternary complex with a formation constant of 10^{21.9}. However, copper was often presented as the metal salt which raises the possibility of metal hydrolysis and its effect on observed binding. Also, simplified equilibria sets were used to determine the apparent constant. By net including all known species into the equilibria sets, unwarranted weight may have been shifted to those species that were included. Further, although other groups have reported the existence of a ternary complex, it was a negligible component at the metal concentrations used in the above experiments (1 mm) (4, 5). Thus, the evidence in the literature for a ternary complex remained unclear.

Several groups have proposed that the zinc and copper sites on albumin are separate and independent. Although much of this evidence is based on work with peptide models, several studies have investigated Zn(II) and Cu(II) competition for albumin sites using intact protein (6-9). Goumakos et al. (10) investigated zinc and copper binding to HSA and DSA by equilibrium dialysis. Kolthoff and Willeford (11) measured the effect of zinc on the oxidation of BSA by copper via amperometric titration. Although their results suggest that the addition of copper does not directly affect zinc binding, the metals were not presented as chelates, which introduces considerable uncertainty about the solution chemistry of the metals (12). We have carried out competition experiments using intact protein under conditions where the speciation of both metals is known. Interpretation of data without ambiguities introduced by polymerization and nonspecific interactions permits direct measurement of competition between metals for a given site.

In addition to these studies, we have determined the intrinsic binding constants for Zn(II) and Cu(II) binding to DSA. Although DSA is biochemically similar to other albumins, it has been reported to have very different copper binding activity due to the absence of His-3 (13, 14). Appleton and Sarkar (13) compared the copper binding activity of HSA and DSA and concluded that DSA had no specific binding site for copper. Although Giroux and Schoun (5) reported an apparent constant for copper binding to DSA which was 3 orders of magnitude lower than that for BSA or RSA, these results were obtained with an ultrafiltration system in which equilibria may not have been achieved. We have re-examined Zn(II) and Cu(II) binding to DSA and determined the intrinsic binding constants so that the metal binding affinities of DSA may be accurately compared to other metal binding proteins.

We have found no evidence to support the hypothesis that free histidine is involved in a BSA-metal-histidine ternary complex. Further, Cu(II) and Zn(II) do not compete for sites on albumin, and the nature of Cu(II) and Zn(II) binding sites on DSA closely resembles those for BSA and HSA.

**MATERIALS AND METHODS**

Metal binding was measured with a system in which Zn(II) and Cu(II) are presented to protein as well-defined, low molecular weight
specific Cu(II) and Cu(I) binding to Dog Serum Albumin

Specific Cu(II) Binding to Dog Serum Albumin

Metal-His-BSA Ternary Complex Formation-Metal chelate solutions were prepared and binding experiments were carried out as above, with one exception. The binding of histidine to BSA was measured, rather than the binding of zinc or copper. Metal chelate solutions were prepared as described using nonisotopic metal and 4-helabeled histidine. Measurement of [\textsuperscript{1}H]histidine activity was done by scintillation counting using the [\textsuperscript{14}C]Cl\textsubscript{2} parameters. [\textsuperscript{1}H]Histidine (DuPont NEN; specific activity 51.5 Ci/mmol) was present only as a tracer and had no evidence from spectroscopic and peptide work to support a copper-histidine-albumin ternary complex (2, 3). This possibility was tested using our strategy with BSA as the standard protein. Labeled histidine was used to complex zinc and copper, and the chelate was presented to BSA. The binding data are presented in Fig. 1. There was no evidence for histidine binding to BSA, either when complexed with metal or by itself.

Zinc-Copper Competition-Binding profiles, as Scatchard plots, were obtained for the binding of zinc to BSA in the presence of either Cu(II), or His. In both cases, the data were superimposable with data for zinc binding in the absence of competitor. The data segregated into two linear groups, one pre- and the other post-saturation. Further, the V\textsubscript{max} values in all three cases were identical (V\textsubscript{max}\textasciitilde 1). The calculated intrinsic constants in each case were also the same. This was expected because the intrinsic constant would not be affected by the presence of a competitor.

However, the apparent constant, that which measures observed binding, would be expected to change. If the two metals were binding to the same site, a decrease in observed zinc binding should occur in the presence of copper. However, direct comparison of the Scatchard plots by overlay is difficult due to the superposition of the data sets. This difficulty can be removed by comparing the regression line parameters from the saturation data sets (Table I). The slopes of the regression lines were not significantly different as measured by the F ratio. This conclusion was corroborated by pairwise t-tests. Thus, Cu(II) does not substantially alter the binding of Zn(II).

Zinc and Copper Binding to BSA-The binding profiles for zinc and copper binding to DSA are shown in Figs. 2 and 3. When metal is presented as the histidine chelate, the data could be divided into two linear groups. The intersection of the two regression lines represents the point at which saturation of the highest affinity site class is reached. This point in the case of Cu(II) binding is clearly at V\textsubscript{max} = 1. Interpretation of the Zn(II) Scatchard plot requires more detailed analysis. The intersection of the regression lines is closer to V\textsubscript{max} = 1 than V\textsubscript{max} = 1.0. Fractional binding sites are conceivable and have been suggested in the case of ferritin subunits. However, we have no evidence that this is the case for albumin. Therefore, we have taken the results of the Scatchard plot as the nearest integer
Specific Cu(II) Binding to Dog Serum Albumin

TABLE I
Regression parameters from zinc binding in the presence of competitors

<table>
<thead>
<tr>
<th>Added competitor</th>
<th>Slope (m x 10^3)</th>
<th>y intercept (m^-1 x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-6.8 ± 1.0</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>-6.3 ± 2.3</td>
<td>6.7 ± 1.2</td>
</tr>
<tr>
<td>Copper-histidine</td>
<td>-4.5 ± 2.0</td>
<td>5.0 ± 1.3</td>
</tr>
</tbody>
</table>

* Values reported as the mean ± sample S.D. (n = 3).

Fig. 2. Scatchard plot of Zn(II) binding to DSA. Zinc was presented as the Zn(His)₃ chelate.

Fig. 3. Scatchard plot of Cu(II) binding to DSA. Copper was presented as the Cu(His)₃ chelate.

The steep portion of the plot represents binding to the high affinity site. Preliminary graphical analysis was done to calculate apparent binding constants so that the binding due solely to the high affinity site could be estimated. Binding to this component was visualized by plotting V versus log[M⁺]ₘₐₓ (Figs. 4 and 5). Saturation is indicated by a sigmoidal curve which reaches a plateau at high metal concentration. Demonstration of binding site saturation is critical for correct interpretation of the binding data (23).

Binding studies carried out at pH 5.0, 7.0, and 8.5 suggested possible metal ligands. Histidyl groups alone are sufficient to describe zinc binding, and, while histidine is suggested as a copper ligand, carboxyl residues may also be involved. These are the same liganding groups indicated for Zn(II) and Cu(II) binding to BSA and HSA (1).

The data used to calculate the DSA intrinsic stoichiometric constants are presented in Tables II and IV.

DISCUSSION

By presenting metal as a well characterized chelate, we have developed a general strategy for investigating metal-protein interactions which maintains metal solubility and prevents metal hydrolysis and subsequent polymerization. We have previously reported the intrinsic constants for zinc and copper binding to BSA and HSA (1). We have extended those studies and addressed several issues which have remained unclear.

Previous spectroscopic and potentiometric studies suggested possible metal binding ligands on albumin. In the case of zinc, these included imidazole, thiol, carboxyl, or peptide oxygen (21, 24, 25). For copper, the ligands included imidazole, o-amino, or peptide nitrogen (21, 26–28). These data bring up two considerations which must be addressed regarding the actual metal ligands in the binding site.

First, it is possible that the histidine ligand suggested by our strategy was free histidine used to present metal to the protein.
This may also have been the case in some spectroscopic studies in which free histidine was used to compete metal from the protein. The complex thus visualized would be a histidine-metal-albumin ternary complex. The possibility of such a ternary complex has been raised by previous investigators using spectroscopic and binding data. Although other groups have also reported the presence of a ternary complex, it was a minor binding to BSA, either alone or when presented as part of a metal chelate (Fig. 1). Based on these results, the formation of the HSA-Cu(I1)-His ternary complex by ultracentrifugation and equilibrium dialysis, using [67Cu]- and [14C]His, and equilibria expressions which may not have been complete.

We have taken a simpler approach and measured histidine binding directly, in the presence or absence of metal. Our results clearly indicate that there is no evidence for histidine binding to BSA, either alone or when presented as part of a metal chelate (Fig. 1). Based on these results, the formation of a ternary complex was not included in the equilibria sets used to determine intrinsic binding constants.

The second consideration about evidence for metal ligands in binding sites based on spectroscopic and potentiometric data is that they do not eliminate the possibility that a histidyl residue in a given binding site may be responsible for binding both copper and zinc. Although the precise location of the zinc binding site on albumin has yet to be determined, Goumaks et al. (10) suggested that this site is at an interior position and not at the accepted N-terminal copper site.

**Table III**

<table>
<thead>
<tr>
<th>Equilibrium concentration</th>
<th>Total metal</th>
<th>Ligand</th>
<th>Bound metal</th>
<th>Ionic</th>
<th>Unbound metal</th>
<th>pH</th>
<th>V</th>
<th>LogK^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>x \times 10^3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.804</td>
<td>1.609</td>
<td>0.108</td>
<td>0.677</td>
<td>0.696</td>
<td>6.935</td>
<td>0.09</td>
<td>5.77</td>
<td></td>
</tr>
<tr>
<td>0.965</td>
<td>1.931</td>
<td>0.240</td>
<td>0.702</td>
<td>0.726</td>
<td>6.925</td>
<td>0.19</td>
<td>6.16</td>
<td></td>
</tr>
<tr>
<td>1.069</td>
<td>2.319</td>
<td>0.498</td>
<td>1.052</td>
<td>1.112</td>
<td>6.926</td>
<td>0.40</td>
<td>6.43</td>
<td></td>
</tr>
<tr>
<td>3.218</td>
<td>6.437</td>
<td>0.688</td>
<td>2.286</td>
<td>2.530</td>
<td>6.920</td>
<td>0.55</td>
<td>6.37</td>
<td></td>
</tr>
<tr>
<td>4.828</td>
<td>9.656</td>
<td>0.864</td>
<td>3.413</td>
<td>3.964</td>
<td>6.925</td>
<td>0.69</td>
<td>6.45</td>
<td></td>
</tr>
<tr>
<td>6.437</td>
<td>12.875</td>
<td>0.892</td>
<td>4.574</td>
<td>5.545</td>
<td>6.930</td>
<td>0.71</td>
<td>6.36</td>
<td></td>
</tr>
<tr>
<td>8.047</td>
<td>16.094</td>
<td>0.823</td>
<td>5.731</td>
<td>7.224</td>
<td>6.925</td>
<td>0.66</td>
<td>6.16</td>
<td></td>
</tr>
<tr>
<td>19.313</td>
<td>38.625</td>
<td>1.815</td>
<td>10.913</td>
<td>17.898</td>
<td>6.950</td>
<td>1.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Protein concentration was constant at 1.25 \times 10^{-4} M.
- \( V \) Due to the equilibrium formulation, when \( V \) was greater than 1, a constant could not be calculated.

**Table IV**

<table>
<thead>
<tr>
<th>Equilibrium concentration</th>
<th>Total metal</th>
<th>Ligand</th>
<th>Bound metal</th>
<th>Ionic</th>
<th>Unbound metal</th>
<th>pH</th>
<th>V</th>
<th>LogK^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>x \times 10^3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.804</td>
<td>1.609</td>
<td>0.363</td>
<td>0.402</td>
<td>0.442</td>
<td>6.895</td>
<td>0.23</td>
<td>9.48</td>
<td></td>
</tr>
<tr>
<td>1.287</td>
<td>2.575</td>
<td>0.434</td>
<td>0.454</td>
<td>0.854</td>
<td>6.900</td>
<td>0.28</td>
<td>9.47</td>
<td></td>
</tr>
<tr>
<td>1.609</td>
<td>3.219</td>
<td>0.640</td>
<td>0.560</td>
<td>0.970</td>
<td>6.955</td>
<td>0.41</td>
<td>9.56</td>
<td></td>
</tr>
<tr>
<td>3.218</td>
<td>6.437</td>
<td>1.255</td>
<td>0.0018</td>
<td>1.964</td>
<td>6.910</td>
<td>0.80</td>
<td>10.76</td>
<td></td>
</tr>
<tr>
<td>4.828</td>
<td>9.656</td>
<td>1.101</td>
<td>0.0032</td>
<td>3.727</td>
<td>6.915</td>
<td>0.70</td>
<td>10.28</td>
<td></td>
</tr>
<tr>
<td>6.437</td>
<td>12.875</td>
<td>1.232</td>
<td>0.0033</td>
<td>5.206</td>
<td>6.925</td>
<td>0.79</td>
<td>10.44</td>
<td></td>
</tr>
<tr>
<td>8.047</td>
<td>16.094</td>
<td>1.851</td>
<td>0.0027</td>
<td>6.196</td>
<td>6.885</td>
<td>1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.875</td>
<td>25.750</td>
<td>1.192</td>
<td>0.0055</td>
<td>11.683</td>
<td>6.905</td>
<td>0.76</td>
<td>10.20</td>
<td></td>
</tr>
<tr>
<td>16.090</td>
<td>32.160</td>
<td>2.966</td>
<td>0.0023</td>
<td>13.094</td>
<td>6.905</td>
<td>1.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.313</td>
<td>38.625</td>
<td>2.356</td>
<td>0.0013</td>
<td>19.657</td>
<td>6.930</td>
<td>1.51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Protein concentration was constant at 1.25 \times 10^{-4} M.
- Due to the equilibrium formulation, when \( V \) was greater than 1, a constant could not be calculated.

Since we have determined the intrinsic constants describing copper and zinc binding to albumin, it is possible to specifically examine zinc binding to its high affinity site under various conditions in which the solution composition is known. Thus, it is possible to create models which describe zinc or copper binding in the presence of additional chelators or metals. Our model predicted that, if copper and zinc were binding to the same site or to interacting sites, the competition would result in a decrease in the observed binding constant. This change in the observed binding would manifest itself as a significant decrease in the slope of the first portion of the Scatchard plot. When Cu(His)_2 competes against Zn(His)_2 experimentally, there is a slight change in the slope. However, the same change is observed when an equivalent amount of His is present (Table I).
Further, neither of the apparent decreases in slope was significantly different from the control slope. These results unambiguously indicate that copper and zinc do not share a binding site on serum albumin.

The current model of zinc and copper binding to albumin postulates that the copper site is at the N terminus and the zinc site exists somewhere in the interior (5, 9, 10, 27). This model, first suggested by Kalthoff and Willeford (29), was based on two lines of evidence. First, amperometric titration experiments suggested that copper was not reacting with a sulphydryl group as had been suggested by Klotz (25, 29). Second, copper added to a solution of BSA decreased the amount of dinitrophenylaspartate yield during the Sanger reaction (27). It was subsequently shown by visible and EPR spectroscopy that synthetic copper-N-terminal peptide complexes produce spectra similar to those observed with copper complexes with intact protein (6, 22, 26).

This model seemed to be corroborated by the metal binding activity of DSA. Appienon and Sarkar (13) observed a difference in copper binding activity between HSA and DSA. However, the binding constants were not quantified. Giroux and Schoun (5) later reported a Cu(I)-DSA binding constant which was several orders of magnitude lower than those for BSA and HSA. These constants were apparent, rather than intrinsic, and were determined under conditions where equilibrium may not have been achieved (5). Sequence data of DSA showed that a tryrosyl residue replaces the histidy of position 3 in BSA and HSA (14). Although Rakhit and Sarkar (22) later reported an interaction between copper and the N terminus of DSA at high pH, the absence of a strong copper site was widely assumed.

Our results, however, clearly show that DSA does have a specific high affinity site for copper. While the intrinsic constant for copper binding is an order of magnitude lower to DSA than to BSA or HSA, it is 3–4 orders of magnitude higher than for zinc. These results suggest a copper site on DSA in which the N-terminal residues are only partially responsible for copper binding. Crystal structure information indicates that the N terminus of albumin is flexible and therefore free to move in space (30). Thus, residues in the main body of the protein may be involved. Binding of copper in this manner would explain both the presence of a specific copper site on DSA and the protection from the Sanger reaction seen by Peters (27).

Since the intrinsic constants for Zn(I) and Cu(I) binding to DSA have been determined, the relative affinities of copper to DSA and other chelators can be compared by plotting pM versus pH (Fig. 6). In these plots, a higher pM indicates lower [M⁺] and thus stronger chelation. This comparison shows that both BSA and DSA can compete with glycine and histidine for copper. BSA can also compete with NTA for copper, but only over a narrow range at physiological pH. DSA cannot effectively compete with NTA at any pH. The slight sequence differences among BSA, HSA, and DSA may explain the differences in copper binding to the various albumins seen in vivo and the serum copper distributions observed in vivo when presented as Cu(NTA) (31).

The ability of DSA to effectively compete for Cu(II) with histidine and other amino acids raises interesting questions regarding copper metabolism in dogs (Fig. 6). The absence of a specific site had been used to explain the increased toxicity of copper in dogs relative to other mammals (13, 32, 33). However, the demonstration of a specific high affinity copper site on DSA suggests that it is unlikely that the sensitivity to serum copper seen in dogs is simply a case of a missing copper binding site.

Our strategy for studying metal-protein interactions enables us to determine intrinsic binding constants. Through this strategy, we are able to more clearly define metal binding activity in complex solutions. In this work, we have examined albumin-metal-histidine ternary complex formation and zinc/copper competition for sites on BSA. We have also determined the intrinsic binding constants for zinc and copper binding to DSA. These results demonstrate the importance of the intrinsic constant to fully understand metal-protein interactions.

Acknowledgments—We thank Bruce R. Van Dyke and David Clotpon for their valuable suggestions and criticisms during the preparation of this manuscript.

REFERENCES