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Structure–Volume Relationships: Singular Volume Effects Produced by Cupric Ion–Globular Protein Interaction†

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ABSTRACT: The nature of the volume isotherms produced by the coordination of Cu(II) with ovalbumin and bovine serum albumin differs substantially from the adsorption isotherms produced by these systems. Whereas there was increased binding of Cu(II) associated with a pH increase from pH 5.3 to pH 7.4, the volume isotherms for these systems did not exhibit this type of pH dependence. The volume changes were determined at 30.0 ± 0.001 °C with microdilatometers which could be read to 0.01 μL. The binding isotherms for ovalbumin at pH 5.3 and 7.4 and for bovine serum albumin at pH 5.3 was resolved by a Scatchard plot to yield the appropriate thermodynamic parameters. An algorithm was derived to calculate the distribution of the individual PM_i complexes, i.e., $PM_{i-1} + M \rightleftharpoons (K_i)\ PM_i$ where i equals 1, 2, 3, ..., n moles of cation, M, bound per mole of protein, P, for the above systems. The volume isotherms were then resolved in terms of the constituent ΔV_i terms, i.e., the volume change produced by the formation of the individual PM_i complexes. These values were verified by an independent graphical differentiation procedure. The coordination of Cu(II) to BSA at pH 7.4 produced a cooperative adsorption isotherm which was not amenable to a Scatchard analysis. The resultant anomalous volume isotherm was resolved into a component related to Cu(II)–site interaction and a negative volume effect attributable to a conformational change induced by complex formation. This structural transition which occurs at physiological pH may constitute a control mechanism for regulating the serum level of Cu(II) and possibly other divalent ions.

The conversion of an apometalloenzyme from the inactive to active form by combination with a specific metal ion effector is considered to be a consequence of a structural transition engendered by the coordination process. The mechanism of this conversion is a matter of conjecture. Insight with respect to the mechanism of this activation process can be gained from dilatometric studies which may provide information pertinent to the type of ligands involved, the change of hydration states of the reactants, and the possible occurrence of conformational changes. A necessary prerequisite is to establish a frame of reference, namely, by determining the volume effects produced by nonspecific binding, i.e., the ΔV[1] resulting from metal ion interaction with a protein. This consideration prompted this dilatometric study of the coordination of Cu(II) with BSA and ovalbumin as a function of pH. To interpret these volume effects, we have determined the volume changes produced by complexing of Cu(II) to specific ligands and to the peptide backbone.

Ovalbumin, M_r 45 040, and BSA, M_r 66 210, were selected because they are proteins with isoionic points in the region of pH 5 (Katz & Ellinger, 1963). These water-soluble proteins differ in several respects: ovalbumin contains about two phosphoserine groups and the N-terminal amino residue is blocked by an acetyl group (Marshall & Neuberger, 1972). Dilatometric titration of this protein by H^+ and OH^- produced volume isotherms which did not exhibit any demonstrable structural transitions in the pH region of 2–11 (Katz & Miller, 1971a). BSA, on the other hand, upon dilatometric titration gave evidence for at least three structural transitions as a

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[1] Abbreviations used: ΔV, volume change; BSA, bovine serum albumin; ΔV_{exp}, experimentally determined volume change; ΔV_{23}, volume change produced by binding of solute, component 3, to protein, component 2; $(Cu_0)/(\text{protein})$, the number of moles of cupric ion added per mole of protein; ν, mean number of moles of cation bound per mole of protein as determined experimentally; n, total number of binding sites in a protein; pI, isoelectric pH; z^+ and z^-, anionic and cationic charge; S, siemens, a unit of conductivity.

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function of pH (Katz et al., 1973).

One of the objectives of this study was to ascertain the feasibility of devising a compouter program capable of resolving the experimentally determined volume isotherms in terms of ΔV_i, the volume change produced by the formation of an individual PM_i complex, i.e., $PM_{i-1} + M \rightleftharpoons (K_i)\ PM_i$, where i equals 1, 2, 3, ..., n. This in turn required a determination of the molecular distribution of the several PM_i complexes as a function of ν. This entailed performing equilibrium dialysis studies in order to determine the number of binding sites and their affinity constants. The ΔV_i terms employed in conjunction with existing thermodynamic data permits one to deduce the nature of the ligands which comprise a given binding site and with this background establish the mechanism of the coordination process. In one system a conformational change occurred; the volume data was resolved in terms of the contribution attributable to metal ion–ligand interaction and that due to the structural transition.

It was established that the interaction of Cu(II) with ovalbumin at pH 5.3 and 7.4 and also with BSA at pH 5.3 produced conventional binding and volume isotherms which were amenable to the above analyses. From inspection of the ΔV_i data for ovalbumin, it was apparent that the binding sites for the first 2 mol of Cu(II) for ovalbumin differed substantially from that of the other binding sites in this protein. The coordination of Cu(II) to BSA at pH 7.4 produced a cooperative binding isotherm and a resultant curvilinear volume isotherm which differs substantially from the previous systems. This indicates that cupric ion can produce a structural transition similar to the alkaline-induced neutral transition (Harmsen et al., 1971). This suggests that this Cu(II)-generated transition may constitute a control mechanism to ensure that the concentration of Cu(II) and conceivably other divalent cations is maintained at normal physiological levels.

Experimental Procedure

Materials. Bovine plasma albumin and ovalbumin were purchased from Sigma Chemical Co. Proteins, pH 7.4, were prepared by adding cold 0.1 M NaOH to a concentrated protein solution until the pH was ~7.6; thin-film dialysis against ice-cold distilled deionized water was performed until the pH dropped to 7.4 (Katz et al., 1980). Samples of dialyzed 10% ovalbumin and BSA were analyzed for Ca(II), Cu(II), Mn(II), Zn(II), Sr(II), Fe(III), and Na(I) by atomic absorption analysis by Dr. John Strohl, Chemistry Department. The concentration of divalent ions was $\leq 5 \times 10^{-5}$ mol/L of solution. The water was passed through two mixed-bed ion exchangers and an activated carbon exchanger, Continental Co., prior to distillation in a glass still; the conductivity was ≤ 0.8 μS. It registered a negative dithizone test.

Methods. The volume changes were determined with Teflon-sheathed microdilatometers at 30.0 ± 0.001 °C. A 8:4 mixing protocol was employed, i.e., 8.00 mL of Cu(II) was pipetted into one arm of the dilatometer and 4.00 mL of 5–8% protein was pipetted into the other. Steady-state volumes were reached 2–3 min after mixing; the data reported are the mean of the values taken at 5-min intervals for the initial 30 min period. Duplicate measurements using different dilatometers positioned in different sections of the thermostat were performed to eliminate systematic errors. The standard deviation of the data is ±0.03 μL or ±4%, whichever is applicable. The details of the methodology have been described (Katz & Ferris, 1966; Katz, 1972).

Equilibrium dialysis was performed with dialysis tubing, molecular weight cutoff of 8000, which was batch washed until the conductivity of the wash water was ≤ 2 μS. The procedure

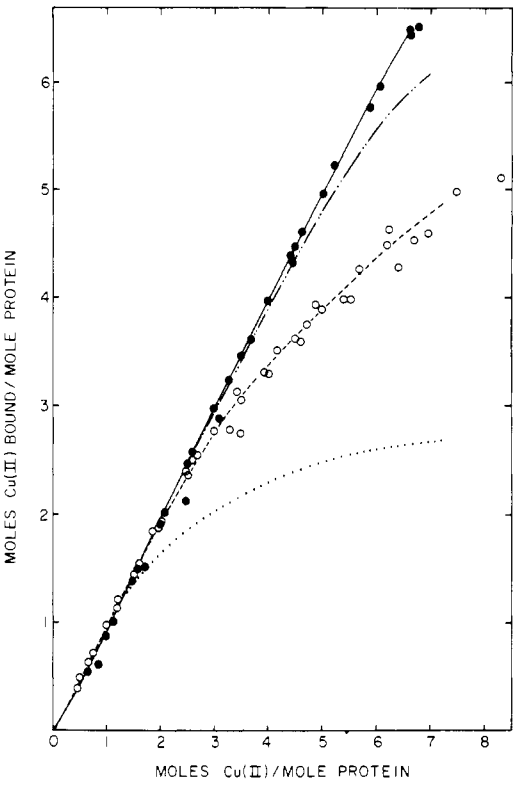

FIGURE 1: Correlation between the number of moles of Cu(II) bound per mole of protein with the number of moles of Cu(II) added per mole of protein. Ovalbumin at pH 5.3 (···); ovalbumin at pH 7.4 (–··–); BSA at pH 5.3 (O---O); BSA at pH 7.4 (●—●).

simulated that used in dilatometry except that 2.00 mL of protein was introduced into the sac and 8.00 mL of Cu(II) was used as the external solution. The initial concentrations of copper ranged from 1×10^{-5} to 5×10^{-3} M. The equilibrium period was 18 h at 30 °C. When the initial Cu(II) concentrations were $\leq 8 \times 10^{-4}$ M, the correction for Cu(II) binding to the sac would amount to ≤20%. The concentration of the stock solutions were determined by EDTA titration. The EDTA solutions were standardized against 99.9% copper wire dissolved in nitric acid (Schwartzenbach & Flaschka, 1969); the experimental and calculated values agreed to within ±0.2%. Cu(II) concentrations from the dialysis experiments were determined with a Jarrel-Ash atomic adsorption spectrophotometer 303. Three calibration curves were used to encompass the concentrations found for these systems. The protein concentrations were determined by dry weights, 18 h at 100 °C in vacuo. These were checked with Gilford-modified Beckman DU2-2 spectrophotometer by employing the value of $A_{279}^{1\%} = 6.67$ for BSA (Leonard et al., 1963). The pH was measured with a Radiometer pH meter, Model 26, with combination electrodes GK2321C standardized with Harleco buffers. Conductivity was determined with a Radiometer conductivity meter, Model EDM3, with a CDC cell. The programs for reducing the data were written in Fortran IV and were executed on a Digital Equipment PDP11/34 computer.

Results

The procedures for the binding isotherms was designed to emphasize precision at the lower binding regions because this is where subtle structural changes would occur as a consequence of binding. Inspection of the adsorption isotherms revealed that Cu(II) binding is a function of the individual protein and the pH of the system; both proteins exhibited an

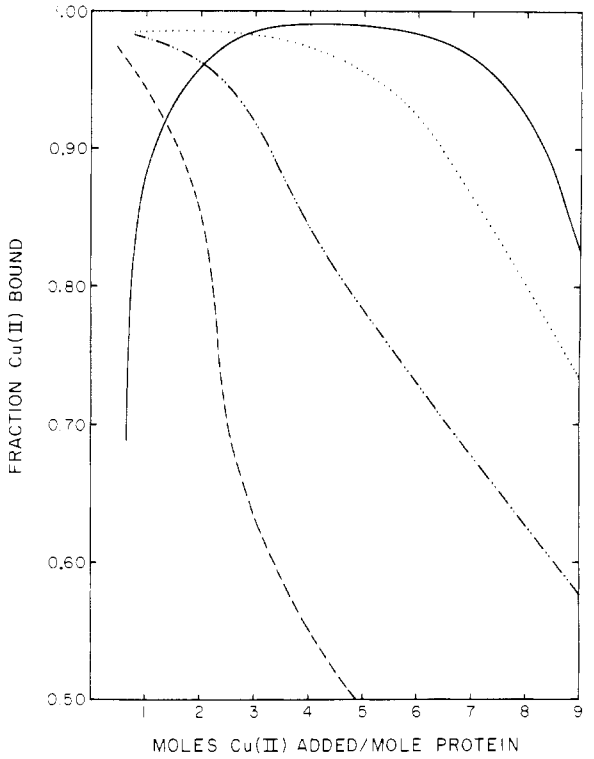

FIGURE 2: Fraction of bound Cu(II) plotted as a function of number of moles of Cu(II) added per mole of protein. Ovalbumin at pH 5.3 (---); ovalbumin at pH 7.4 (···); BSA at pH 5.3 (–··–); BSA at pH 7.4 (—).

increase of binding with increasing pH (Figure 1). It is apparent that the binding capacity of BSA is substantially greater than that of ovalbumin and that this difference is most pronounced in the vicinity of the isoelectric point. The binding isotherm for ovalbumin could be resolved by a Scatchard (1949) plot to yield two sets of sites: n_1 was 1.9 with k_1 of 8.75×10^4 and n_2 was 1.6 with k_2 of 3.5×10^3. At pH 7.4 the corresponding terms were n_1 of 4 and k_1 of 17.0×10^4 and n_2 of 4 and k_2 of 2.0×10^4. For BSA at 5.3 we obtained values of n_1 of 3.5, k_1 of 8.1×10^4, n_2 of 2.5, and k_2 of 9.0×10^3. One observes that ovalbumin at pH 7.4 is characterized by having about double the number of sites observed at pH 5.3 and the values for the k_i terms are substantially larger than those determined at pH 5.3.

The interaction of Cu(II) with BSA at 7.4 produced a binding pattern which differed substantially from the aforementioned systems by being cooperative in nature. Consequently, the conventional Scatchard analysis was not applicable, and since the saturating level of binding was not attained, a Hill plot could not be constructed (Dahlquist, 1978). The difference between the isotherm for Cu(II)–BSA at pH 7.4 and isotherms for the other systems is demonstrated by comparing the value of F, the ratio of ν to the total number of moles of Cu(II) added to the system, $\nu/[Cu_0]$, as a function of the ratio of the total number of moles of Cu(II) added per mole of protein, $[Cu_0]/[\text{protein}]$. For BSA at pH 5.3 at values of $[Cu_0]/[\text{protein}]$ of 0.75, 1, 2, 3, 4, and 5, the values for $\nu/[Cu_0]$ were 0.98. 0.98, 0.96, 0.92, 0.84, and 0.78. Whereas for BSA at pH 7.4 the corresponding terms were 0.82, 0.87, 0.96, 0.98, 0.99, and 0.99 (Figure 2). The absence of systematic error is attested by the conventional isotherms produced by ovalbumin at pH 5.3 and 7.4. Since there exist studies relating proton release as a function of Cu(II) binding, there was no need to repeat this (Gurd, 1970; Reynolds et al., 1973). However, one notes that at pH 5.3 when ν was 5 the

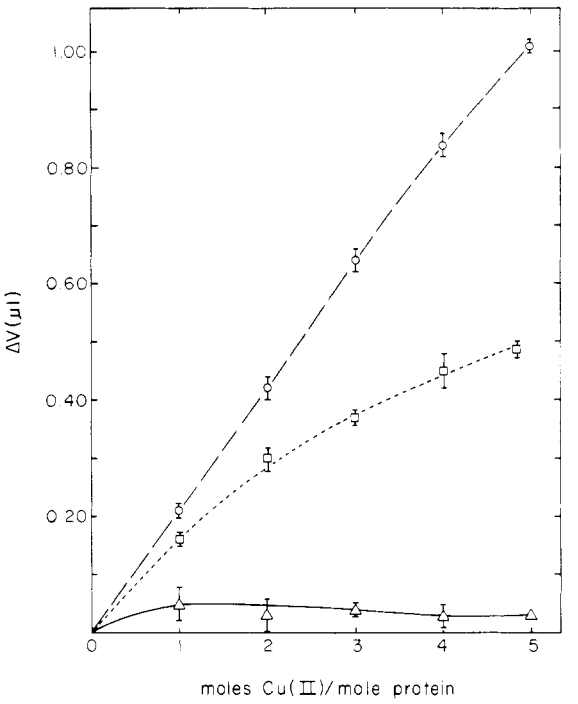

FIGURE 3: Comparison of dilatometric data produced by the addition of Cu(II) to several systems (for details refer to Experimental Procedure). 5% BSA at pH 5.3 (○—○); 5% ovalbumin at pH 5.3 (□---□); H_2O (△—△).

ΔpH for ovalbumin was –0.46 whereas for BSA this function was –0.72. At pH 7.4 the ΔpH isotherms for these two protein were essentially similar.

To discuss the volume data, it is necessary to define several terms. The volume change produced by the binding of ν moles of solute, component 3, per mole of protein, component 2, is given by the expression:

$$\Delta V_{23} = (\Delta v_{23} - \Delta v_{13} - \Delta v_{12})/n_2$$

where the term Δv_{23} is the experimentally observed volume change produced by the coordination of ν moles of solute 3 to n_2 moles of protein, Δv_{13} is a volume change produced by the identical system except that the protein is omitted, and Δv_{12} is a volume change for the dilution of the protein in this system. It has been shown that the value for Δv_{12} is negligible because the partial molar volume of protein is constant at concentrations ≤20% (Katz & Ferris, 1966; Bernhardt & Pauly, 1975). By reference to Figure 3, it is apparent that the value for Δv_{13} is small, the maximum magnitude being ~0.04 μL. In this study, since ≥60% of the added cupric ion is bound to the protein, this correction can be neglected. Consequently, the following expression was used:

$$\Delta V_{23} = \frac{\Delta v_{23}}{n_2}$$

The ΔV_{23} isotherms produced by the formation of Cu(II)–protein complexes are a unique function of the individual protein and exhibit no characteristics in common with the corresponding binding isotherms (compare Figures 1 and 4). It is evident that the pH dependence of these volume effects is a function of the individual protein. Upon closer inspection it was noted that these isotherms could be classified into two categories: the volume isotherms for ovalbumin at pH 5.3 and 7.4 and for BSA at pH 5.3 can be considered as consisting of two straight segments whereas Cu(II)–BSA at pH 7.4 produced volume effects which were smaller in magnitude than the aforementioned systems and the resultant isotherm obeyed

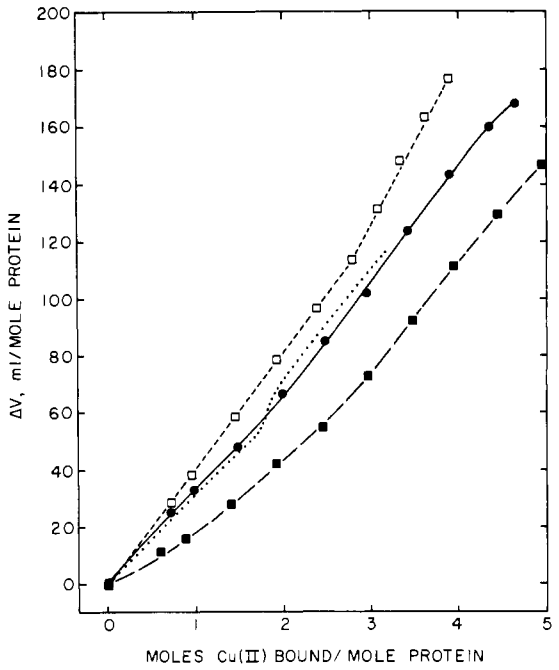

FIGURE 4: Volume change (milliliters per mole of protein) produced by coordination of Cu(II) to protein. Ovalbumin at pH 5.3 (···); ovalbumin at pH 7.4 (●—●); BSA at pH 5.3 (□- - -□); BSA at pH 7.4 (■- - -■). Only a few of the experimental points are represented.

positive curvilinear relationship with Cu(II) concentrations (Figure 4).

The volume isotherms for Cu(II)–ovalbumin at pH 5.3 and 7.4 are similar in magnitude with curves exhibiting a break of slope in the region where ν is 1.9. The increase of magnitude of ΔV_{23} at pH 5.3 at the point of inflection may be an artifact attributable to the diminution of the slope of the binding isotherm in this region (Figures 1 and 2). To illustrate the magnitude of these volume effects, let us consider the systems where ν is 3; for ovalbumin at pH 5.3 and 7.4, values of 110 and 105 mL were observed. For BSA the corresponding data were 125 and 73 mL/mol for the system at pH 5.3 and 7.4. It is apparent that the mechanisms involved in generating the volume change for BSA at pH 7.4 must differ substantially from that operational for this protein at pH 5.3 (see Discussion).

Discussion

One of the objectives of this study was to establish the feasibility of determining values for ΔV_i, the volume change produced by the formation of a given PM_i complex by analysis of the volume isotherm–ν functional dependence. The approach adopted was to consider the volume change produced by the system:

$$M + P \underset{}{\overset{K_1}{\rightleftharpoons}} PM \qquad \Delta V_1 \qquad (1)$$

$$M + PM \overset{K_2}{\rightleftharpoons} PM_2 \qquad \Delta V_2 \qquad (2)$$

$$\vdots \qquad\qquad \vdots \qquad\qquad \vdots$$

$$M + PM_{n-1} \overset{K_n}{\rightleftharpoons} PM_n \qquad \Delta V_n \qquad (3)$$

The terms ΔV_1, ΔV_2, ..., ΔV_n represent the volume effects produced by each of the corresponding reactions. The fraction of the total protein existing as a given PM_i complex, f_i, can be calculated from the relationships (Klotz, 1953; Weber, 1975)

$$f_i = \frac{(PM_i)}{(P) + \sum_1^n (PM_i)} \qquad (4a)$$

or

$$f_i = \frac{\prod_1^i K_i(M)^i}{(P) + \sum_1^n \prod_1^i K_i(M_i)^i} \qquad (4b)$$

The values in parentheses indicate concentrations and K_i represents the stoichiometric affinity constants. If it is assumed that there are S sets of binding sites which can be classified into n_i subsets with each subset consisting of essentially equivalent noninteracting residues with intrinsic affinity constants, k_i, we can derive the equation (Klotz, 1953; Edsall & Wyman, 1958)

$$\nu = \sum_1^S \frac{n_i k_i(M)}{1 + k_i(M)} \qquad (5)$$

The term ν is equivalent to the mean number of metal ions bound per mole of protein. The relationship between K_i and k_i is

$$K_i = (n - i + 1)k_i/i \qquad (6)$$

To determine ΔV_i from the experimental volume isotherm, it is assumed that ΔV_{23} is the sum of the ΔV_i terms resulting from the formation of the several PM_i complexes as a function of concentration; that is

$$\Delta V_{23} = (f_1 + f_2 + f_3 + \ldots + f_n)\Delta V_1 + (f_2 + f_3 + \ldots + f_n)\Delta V_2 + \ldots + f_n \Delta V_n \qquad (7a)$$

or

$$\Delta V_{23} = \sum_1^n \sigma_i \Delta V_i$$

where

$$\sigma_i = \sum_{j=i}^n f_j \qquad (7b)$$

To determine the distribution of PM_i complexes as a function of Cu(II) concentration, the values for K_i were calcuated by using eq 6, employing values of n_i and k_i evaluated from the corresponding binding isotherms. An algorithm was derived to calculate ν_i and f_i as a function of cupric ion concentration by employing eq 4b and 5. To facilitate the calculations, the values for n_i, when necessary, were adjusted to the nearest integral value. This in turn might require reducing the value of k_i by ≤10%; the agreement between the experimental and calculated ν isotherms was within ±5% for the initial 75% of the curves.

At this point reference to the distribution of the PM_i complexes as a function of ν is helpful (Figures 5–7). It is apparent that, when the log $(K_i/K_{i+1}) < 1.5$, several forms of PM_i, complexes will coexist at a given value of ν. Also, for most systems a sizable amount of the protein will exist in the uncomplexed state at $\nu \leq 1.5$. Let us consider the distribution of the PM_i complexes as influenced by the selection of the values of n_i and k_i. The distribution of PM_i complexes, subject to the constraint that $\nu \ll n$, is not affected markedly by the choice of values of n and k_i, i.e., within reasonable bounds. For example, consider the distribution of the PM_i complexes where $n_1 = 4$, $k_1 = 8 \times 10^4$, $n_2 = 3$, and $k_2 = 8.9 \times 10^3$ (BSA at pH 5.3) with a similar system where $n_1 = 4$, $k_1 = 17 \times 10^4$, $n_2 = 4$, and $k_2 = 1.95 \times 10^4$ (ovalbumin at pH 7.4) (Table I). The composition of these two systems with respect to the concentration of complexes formed are similar even though the magnitude of k_i differs by a factor of 2. However, when

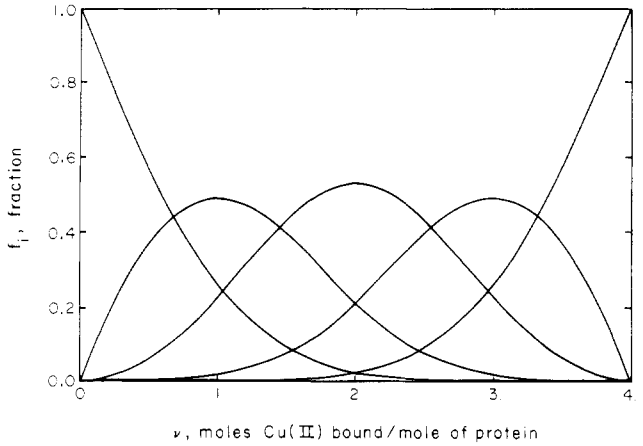

FIGURE 5: Distribution of fraction of metal ion–protein complexes, f_i, formed in a given system as a function of ν. This depicts the molecular distribution for Cu(II)–ovalbumin complexes, pH 5.3, as function of ν. These curves were derived by the use of eq 4; the parameters employed were $n_1 = 2$, $k_1 = 8.75 \times 10^4$, $n_2 = 2$, and $k_2 = 3.55 \times 10^3$. The curve at the extreme left, top of graph, represents the protein, while the curves starting at the left are the 1:1, 2:1, ..., n:1 Cu(II)–ovalbumin complexes.

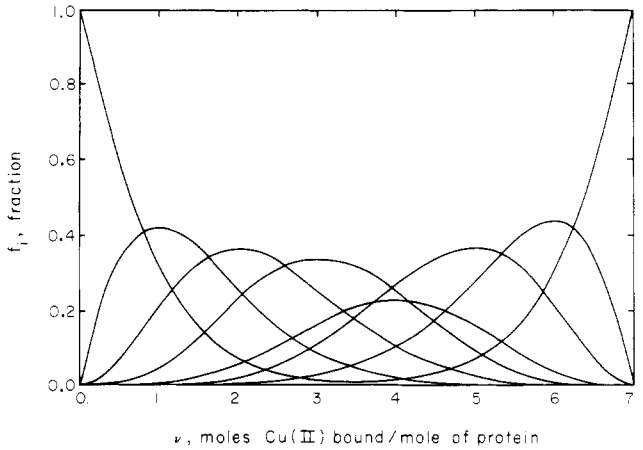

FIGURE 6: Distribution of Cu(II)–BSA, complexes, pH 5.3, as a function of ν. The parameters employed were $n_1 = 4$, $k_1 = 8 \times 10^4$, $n_2 = 3$, and $k_2 = 8.9 \times 10^3$. See Figure 5 for details.

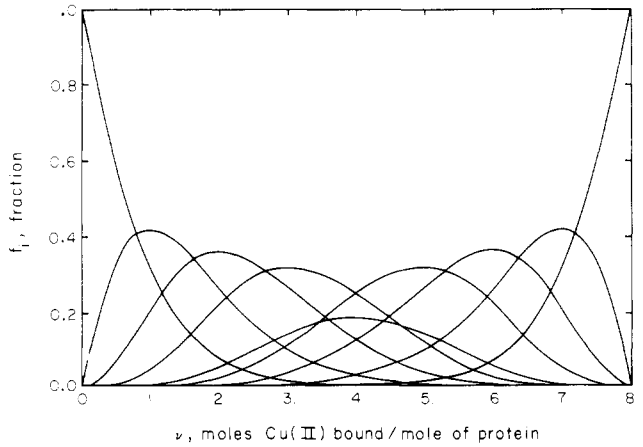

FIGURE 7: Distribution of Cu(II)–ovalbumin complexes, pH 7.4, as a function of ν. The parameters employed were $n_1 = 4$, $k_1 = 17.1 \times 10^4$, $n_2 = 4$, and $k_2 = 1.9 \times 10^4$.

ν approaches n, then there was a substantial difference in the distribution of the PM_i components, e.g., at $\nu = 6.9$ the values for a given PM_i complex can differ by several orders of magnitude. It is apparent that a check of validity of the values

Table I: Distribution of Protein–Ion Complexes as a Function of Binding Parameters

components	seven sites[a]		eight sites[b]	
	ν	fraction	ν	fraction
	2.029		2.003	
M		0.0012		5.4×10^{-4}
P		6.557		7.12
PM_1		25.338		26.30
PM_2		36.715		36.43
PM_3		23.646		22.42
PM_4		5.7105		5.182
PM_5		1.830		2.183
PM_6		0.195		0.345
PM_7		0.007		0.002
PM_8				6.4×10^{-4}
	6.88		6.89	
M		0.2741		0.0135
P		2.6×10^{-12}		2.0×10^{-8}
PM_1		2.3×10^{-9}		0.0002
PM_2		7.5×10^{-7}		0.0064
PM_3		1.1×10^{-4}		0.099
PM_4		0.0061		0.573
PM_5		0.447		6.03
PM_6		10.90		23.86
PM_7		88.65		41.87
PM_8				27.56

[a] BSA, pH 5.3: $n_1 = 4$; $k_1 = 8.05 \times 10^4$; $n_2 = 3$; $k_2 = 8.9 \times 10^3$. [b] Ovalbumin, pH 7.4: $n_1 = 4$; $k_1 = 17.1 \times 10^4$; $n_2 = 4$; $k_2 = 1.95 \times 10^4$.

for n and k_i is by determining the correlation existing between the experimental and calculated ν isotherms as a function of unbound Cu(II).

Since there exists a wealth of reviews of the metal ion–protein interaction (Gurd, 1970; Breslow, 1973; Sundberg & Martin, 1974; Sarkar, 1977), this section will be limited to those factors of direct relevance for interpreting the observed volume effects. Copper can complex to carboxylate residues, nitrogen donor atoms, and the peptide backbone (Nakon & Angelici, 1974; Katz & Shinaberry, 1978). It has been established that the N-terminal peptide in BSA is one of the primary binding sites of Cu(II), the cation being coordinated to the terminal α-amino group, histidine-3, and two intervening peptide bonds with displacement of two or more protons as a function of pH (Peters & Blumenstock, 1967). The N-terminal group of ovalbumin is blocked by an acetyl group (Marshall & Neuberger, 1972); consequently, binding to the N-terminal portion of ovalbumin must differ from that of serum albumin. Buffers and supporting electrolytes were avoided to preclude competitive binding effects, mixed complex formation, i.e., protein–metal ion–buffer constituents, and other less well-defined buffer anomalies [refer to Katz & Squire (1980) for details]. Supporting electrolytes which are often used to suppress Donnan effect were avoided to preclude the formation of protein–anion complexes which are concentration and pH dependent. Ionic dissymmetry at pH 5.3 for these proteins is negligible because of electroneutrality considerations. At pH 7.4 the Donnan contribution $\leq 15\%$; this is based on unpublished studies with calcium ion (Katz, 1953). The binding of a divalent cation to a protein at a pH > pI will reduce the electrostatic charge of the protein–metal ion complex, thereby reducing the Donnan contribution. Inspection of the binding data for BSA at pH 7.4 (Figure 2) reveals that the Donnan contribution is small relative to the effect of binding.

The cooperative binding isotherm produced by Cu(II)–BSA complex formation at pH 7.4 may be a manifestation of the structural transition induced by this coordination process. This

phenomenon may be similar in kind or may be identical with the alkali-induced neutral transition (Harmsen et al., 1971). One manifestation of the neutral transition is the enhanced binding of calcium to BSA at pH values ≥7.0 substantially exceeding that ascribed to charge effects and was interpreted as being a consequence of a structural transition (Katz & Klotz, 1953; Zurawski & Foster, 1974).

For evaluation of these volume effects, a review of the volume changes produced by Cu(II)-specific ligands is in order. The addition of Cu(II) to acids, i.e., carboxylics, ammonium, imidazolium, etc., produces no discernible volume effect because complexation is negligible (Katz et al., 1975). The formation of a 1:1 Cu(II)–carboxylate complex results in a ΔV of 13 ± 1 mL/mol which is not influenced markedly by alkyl substituents (Katz et al., 1975). The coordination of Cu(II) or Zn(II) to nitrogen donor atoms such as amines and histidyls evokes a volume increase of 2–4 mL/mol (Katz et al., 1978). The process of forming a cyclic structure, i.e., chelate formation involving Cu(II) and carboxylate or amines, generates small volume changes, i.e., 0–2 mL/mol of chelate formed. Copper can form at least two types of chelates with dipeptides as a function of pH (Nakon & Angelici, 1974). In systems acid to neutrality, Cu(II) forms a bidentated chelate involving the terminal nitrogen donor atom and the carbonyl oxygen of the dipeptide to form a positively charged cationic complex; the ΔV is 11 mL/mol (Katz & Shinaberry, 1978). At pH values alkaline to neutrality, a tridentated chelate is formed with copper coordinating to the terminal amine, the nitrogen of the peptide backbone, and the carboxylate oxygen; a proton is expelled from the backbone peptide as a consequence of this reaction. The ΔV for this process is 20 mL/mol, with ~3 mL/mol of this effect attributed to the configurational transformation and expulsion of a proton from the peptide nitrogen.

The protonation of carboxylates and nitrogen donor atoms produces volume changes and thermodynamic parameters which tend to parallel those of Cu(II) coordination processes. The protonation of a carboxylate group results in a volume increase of ~11 mL/mol (Kauzmann et al., 1962). The combination of a proton to nitrogen donor atoms causes small negative volume changes; e.g., for imidazole it is −1.7 mL/mol and for primary amines the ΔV is about −2 mL/mol (Katz & Miller, 1971b).

The volume changes produced by cation–ligand coordination can be related to the appropriate thermodynamic parameters; this correspondence can be used as a predictive tool. The coordination of the Irving & William (1948) series of cations with carboxylates are exergonic reactions driven by the large positive entropy change; the enthalpy contribution is a small positive value (Angelici, 1973). The combination of these cations to nitrogen donor atoms generates a sizable negative enthalpy change, whereas the entropy contribution is small. A similar correspondence exists in the volume effects: i.e., when Cu(II) coordinates to nitrogen donor atoms, the volume effects are small; this is to be anticipated from the small entropic changes for these systems (Katz et al., 1975). However, the coordination of cupric ion to ligands incorporating oxygen, namely, carboxylates and carbonyl oxygens, results in large volume effects; these are systems characterized by large positive entropy changes. It is proposed that the large volume and entropy changes are a consequence of the release of water of hydration upon coordination (Katz et al., 1975). A somewhat similar conclusion based on the substitution of water into cobalt complexes has been proposed by Spiro et al. (1968). The same relationships apply also to protonation of

Table II: Values of ΔV_i Produced by Cu(II)–Protein Complex Formation[a]

	ovalbumin		
	pH 5.3[b]	pH 7.4[c]	BSA, pH 5.3[d]
ΔV_1	30	31	39
ΔV_2	32	33	40
ΔV_3	44	39	42
ΔV_4	–	40	60
ΔV_5		42	62
ΔV_6		–	–
ΔV_7		–	–
ΔV_8			–

[a] For details of calculations refer to eq 7. $Cu(II) + Cu_{i-1}P \rightleftharpoons (K_i)\ Cu_iP$. [b] $n_1 = 2; k_1 = 8.75 \times 10^4; n_2 = 2; k_2 = 3.55 \times 10^3$. [c] $n_1 = 4; k_1 = 1.71 \times 10^4; n_2 = 4; k_2 = 1.95 \times 10^4$. [d] $n_1 = 4; k_1 = 8 \times 10^4; n_2 = 3; k_2 = 8.9 \times 10^3$.

carboxylates and nitrogen donor atoms (Katz & Roberson, 1976). X-ray crystallographic studies reveal that water is an integral feature of protein structure and tends to be localized in the region of oxygen atoms. Carboxylate groups have more hydrogen bonded water than nitrogen donor atoms such as lysine. Similarly, the carbonyl oxygens in the peptide backbone characteristically had more water bonded than did the N–H groups (Watenpaugh et al., 1978). These data lend credence to the hypothesis that coordination to an oxygen donor atom will release more water than the corresponding process involving nitrogen donor atoms.

The volume effects produced by combination of a metal salt, $M^{z+}(H_2O)_aX^{z-}(H_2O)_b$, with a protein containing several subsets of binding sites is represented by eq 8. The symbols

$$n\mathrm{M}^{z+}(\mathrm{H_2O})_a + \alpha n\mathrm{X}^{z-}(\mathrm{H_2O})_b +$$

$$\begin{array}{l|c|l} (Z_i) & & (Z_f) \\ \hline -n_1S_M'(H_2O)_c & & -n_1S_M'(H_2O)_{c-j'}M(H_2O)_{c-y'} \\ -n_2S_M''(H_2O)_d & & -n_2S_M''(H_2O)_{a-j''}M(H_2O)_{d-y''} \\ -p_1S_X'(H_2O)_e & \rightleftharpoons & -p_1S_X'(H_2O)_{e-z'}X(H_2O)_{b-u'} + \\ -p_2S_X''(H_2O)_f & & -p_2S_X''(H_2O)_{f-z''}X(H_2O)_{b-u''} \\ -q_1B_1(H_2O)_g & & -q_1B_1(H_2O)_{g-u'}H(H_2O)_{g-r'} \\ -q_2B_2(H_2O)_h & & -q_2B_2(H_2O)_{n-u''}H(H_2O)_{g-r''} \end{array}$$

$$\Sigma\phi_i(\mathrm{H_2O}) \quad (8)$$

employed for the salt are self-explanatory, whereas for the protein the lower-case letters refer to the number of sites and the upper-case letters refer to binding sites with different affinities for cations, S_M, anions, S_X, and protons, B_i. The amount of water released, $\phi_i(H_2O)$, is a function of the concentration and types of constituents present. The volume change produced (see eq 9)

$$\Delta V = \Delta V_{PM_i} + \Delta V_{PA_g} + \Delta V_{PMH} + \Delta V_{conf} + \Delta V_{MX,dil} + \Delta V_{P,dil} \quad (9)$$

is determined primarily by the following factors: ΔV_{PM_i}, the volume effects due to the formation of a protein–metal ion complex; ΔV_{PA_g}, the volume effect associated with the formation of a protein–anion complex; ΔV_{PMH}, the volume effects associated with the gain or loss of protons; ΔV_{conf}, the volume effect due to conformational changes in the protein; $\Delta V_{MX,dil}$, the volume effect produced by the dilution of the salt; $\Delta V_{P,dil}$, the volume change due to the dilution of the protein upon mixing. For most systems the observed volume change is due primarily to three factors: the formation of protein–metal ion complexes, the change of the protonation state of the protein, and the conformational changes generated by the coordination process. The contribution due to anion binding for these

systems is small; the ΔV produced by the binding of both inorganic and organic anions at concentrations ≤ 0.01 M was ≤ 30 mL/mol of BSA (Katz et al., 1974). The contribution due to $\Delta V_{MX,dil}$ and $\Delta V_{P,dil}$ can be neglected for reasons presented previously.

At this stage we can proceed to evaluate ΔV_i by employing eq 7. The linear set of equations obtained proved to be "ill conditioned" and not amenable to conventional analysis.[2] Consequently, a "peeling" technique was used to calculate the roots where $v < 1$; this involved solution of matrices of the rank of two. Then sets of equations with progressively larger values for ΔV_{23} were solved by successive approximations. The results obtained are summarized in Table II. Two independent avenues were used to assess the validity of the values. One approach was to reconstruct the ΔV_{23} isotherm as a function of the f_i and ΔV_i terms. The other approach was to graphically differentiate the experimental volume isotherms by the chord–area method (Klotz & Rosenberg, 1972); this yields $\partial \Delta V_{23}/\partial \nu$, the partial derivative of the volume change with respect to ν as a function of ν. It is apparent by referring to Figures 5–7 that these partial derivatives can be related to the appropriate values of ΔV_i terms as a function of ν.

The analysis of the volume effects produced by the Cu(II)–ovalbumin system at pH 5.3 revealed the existence of two sets of sites. The first set was characterized by values of ΔV_1 and ΔV_2 of 30 and 32 mL/mol; for the next set we calculate a ΔV_3 of 44 mL/mol; ΔV_4 is not reported because it is too tenuous. The chord–area analysis of the ΔV_{23} isotherm resulted in a $\Delta(\Delta V)/\Delta \nu$ plot which consisted of two linear sections with a break in the isotherm occurring where $\nu = 1.85$. The slope of the first segment was 30.5 mL/mol and the second was 40 mL/mol. The results for ovalbumin at pH 7.4 are somewhat surprising because of the difference of the volume isotherms at pH 5.3 and 7.4. There are two sets of sites; the first set of sites yields values of ΔV_1 and ΔV_2 of 31 and 33 mL/mol, respectively, which is virtually identical with that determined at pH 5.3. For the second group the values for ΔV_3, ΔV_4, and ΔV_5 were 39, 40, and 42 mL/mol. The chord–area plot yielded two linear segments with the point of inflection at ν of 1.9; the slopes were 33 and 41 mL/mol, respectively. The close correlation between the values of the ΔV_i and $\partial \Delta V/\partial \nu$ terms (recalling that they have a causal but not linear relationship) is striking and lends credence to the approach used to calculate ΔV_i.

The correspondence between the values for ΔV_1 and ΔV_2 of ~ 31 mL/mol and the ΔV_i terms where $i \geq 3$ of ~ 40 mL/mol at both pH 5.3 and pH 7.4 is singular considering the substantial difference in character of the volume and binding isotherms (compare Figures 1 and 4) and in view of the disparity of the values of n_i derived by the Scatchard analysis. It appears that the ΔV_i terms resulting from Cu(II) binding to ovalbumin sites are the same or similar and exhibit virtually no pH dependence. There are several hypotheses which may account for volume change of 30 mL/mol for the first two sites in ovalbumin. It can be postulated that two carboxylates coordinate to Cu(II), eliciting a ΔV of ~ 26 mL/mol. The remainder could be due to the coordination with the nitrogen donor atoms of histidine, generating an additional 2 mL/imidazole. Another possibility is that these sites represent the two phosphoserines incorporated in ovalbumin. However, because the pK of the phosphate radical and the ΔV for complex formation are unknown, no firm statement can be made. The value for ΔV_i of 40 mL/mol for the other sites can be attributed to Cu(II) coordination to three carboxylates; additional stabilization could be provided by linking the other coordination sites of Cu(II) to histidyl residues.

The values for ΔV_i for the Cu(II)–BSA system at pH 5.3 also fell into two sets with the values for ΔV_1, ΔV_2, and ΔV_3 being 39, 40, and 42 mL/mol while ΔV_4 and ΔV_5 were ~ 60 mL/mol. The chord–area analysis gave slopes of 40.5 and 57.5 mL/mol with the break in the isotherm occurring where ν equalled 2.9; the agreement is considered as being satisfactory. One explanation for ΔV_i of 40 mL/mol, where $1 \leq i \leq 3$, is that the volume effects are due to coordination to the peptide backbone [see Gurd (1970) for a detailed discussion] and to polar groups. Burkhard's group (Reynolds et al., 1973) in a careful study of BSA–Cu(II) interaction at pH 4.8 established that two to three protons were released upon the binding of the first 4 mol of Cu(II) to albumin. According to our previous studies, this process would be analogous to the formation of a tridentated Cu(II)–dipeptide complex with a ΔV of 20 mL/mol of complex formed (Katz & Shinaberry, 1978). At least two models can be proposed to explain the above values, namely, the coordination of Cu(II) to two peptide elements in BSA will cause a resultant ΔV of ~ 40 mL/mol or the second alternative that Cu(II) binds to one peptide segment and to two carboxylate radials to produce a volume change of ~ 40 mL/mol. One notes that the magnitude of ΔV_i where $1 \leq i \leq 3$ for Cu(II)–BSA at pH 5.3 is similar to ΔV_i where $i \geq 3$ for the Cu(II)–ovalbumin system which suggests that similar mechanisms may be involved. One can not dismiss this possibility in a cavalier manner; however, it should be recalled that the ΔpH decrease resulting from the binding of Cu(II) to ovalbumin at pH 5.3 is about half that observed for the comparable serum albumin system.

The volume effects for ΔV_i, where $\lambda \geq 4$, of ~ 60 mL/mol can be explained by several mechanisms. Namely, in the coordination of Cu(II) to two peptides, producing a ΔV of 40 mL/mol, the additional 20 mL/mol increment could result from the protons expelled from the peptide nitrogen combining to two carboxylate groups. At the initial stages of Cu(II) binding, the released protons react with the histidyl residues, producing a neglible effect (Katz & Miller, 1971b). Another mechanism is that Cu(II) coordinates to one peptide backbone and to two or three carboxylates. Even though we can not define the process precisely, it is evident that the large magnitude of these volume effects indicates Cu(II) coordination to oxygen donor atoms because the volume effects associated with coordination to nitrogen donor atoms produce small volume changes.

The volume effects elicited by the complexing of Cu(II) to BSA at pH 7.4 could not be resolved by the use of eq 7 in view of the cooperative character of the binding isotherm (Figure 2). Graphical differentiation of the volume isotherm for Cu(II)–BSA at pH 5.3 yielded two linear segments with a break at ν of 2.9 (Figure 8). This analysis, when applied to the pH 7.4 system, produced a curvilinear relationship for

[2] The initial attempt to solve the set of linear equations by SOLVE (Forsythe et al., 1977) proved to be unsuccessful. The values for ΔV_i exhibited the behavior typical of an ill-conditioned system; i.e., the roots were large and oscillated in sign. This occurs when the determinant for the set of equations is small relative to the magnitude of the individual coefficients. We then applied Singular Value Decomposition using the subroutine SVD (Forsythe et al., 1977); again these equations proved to be extremely ill-conditioned with the ratio of the largest to smallest singular values being of the order of 10^8. Truncating the system, i.e., setting the smaller singular values to zero, improved the quality of the solutions considerably but the periodic fluctuation of the ΔV_i terms indicated that the solutions were not realistic. The ΔV_i terms varied as a function of the number of singular values retained and the selection of Cu(II) concentrations. Consequently, we resorted to a "peeling" technique.

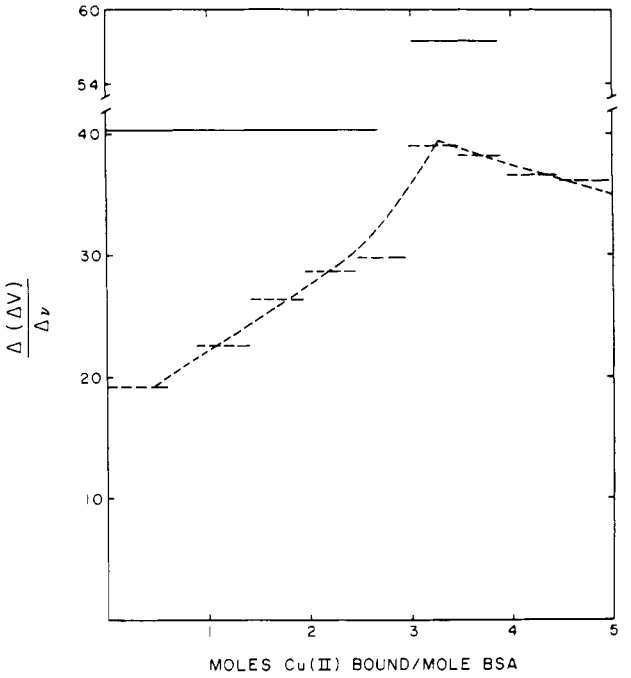

FIGURE 8: Chord–area plot of the ratio of the incremental volume change to the incremental change of ν, $\Delta(\Delta V)/\Delta\nu$, plotted as a function of moles of Cu(II) bound per mole of BSA (see the text for details): BSA, pH 5.3 (—); BSA, pH 7.4 (---).

$\partial\Delta V/\partial\nu$ with this function increasing from 19.5 mL/mol where ν was 0.5 to a maximum of 39.5 mL/mol where ν was 3.2. With an increase of binding, there was a gradual decrease, yielding a value of 35 mL/mol where ν equals 5. The discontinuity in the curve in the region where ν is $\sim$3 agrees with the ΔV_i data for BSA at pH 5.3 (see Table II). Similarly, the parameter, n, derived by the Scatchard analysis of BSA at pH 5.3 was 3.5. Obviously there is a marked alteration of the properties of BSA when 3 or more moles of Cu(II) ions is bound to the protein.

These singular volume effects observed for the pH 7.4 system may be interpreted as follows. If we assume that the ΔV_i values for ovalbumin represent the norm, i.e., the ΔV_i terms at both pH values are essentially the same (Table II), then the values for ΔV_i for BSA at pH 5.3 and 7.4 should be similar. The attenuated volume effects at pH 7.4 result from a structural transition generated by the coordination of Cu(II) to this protein. If the 1:1 and 2:1 copper complexes predominate in systems where $\nu \leq 1$ (see Figures 5–7), the volume effects are the resultant of two phenomena: (1) the ΔV due to the formation of Cu(II)–protein complexes and (2) the ΔV attributable to the conformational change. The volume effects due to the formation of the first few complexes can be assumed to be $\sim$40 mL/mol based on the values for the ΔV_i terms where $i \leq 3$ for Cu(II)–BSA at pH 5.3. Since the value for $\partial(\Delta V)/\partial\nu$ is about −20 mL/mol in the region where $0.5 \leq \nu \leq 1$, the mean conformational contribution for the formation of both the first and second copper complexes of BSA is about −20 mL/mol. This negative volume effect can be attributed to several factors. The α-helix to coil transition results in a ΔV of about −1 mL/mol of amino acid residues (Noguchi & Yang, 1963), while a β-helix to coil transition causes a ΔV of about −2 mL/mol of amino acid residue (Makino & Noguchi, 1971). If we assume that this transition is essentially another manifestation of the neutral transition, the above is irrelevant since there is little change in the secondary structure attributable to this transition (Braam, 1972; Zurawski & Foster, 1974). A more substantial hypothesis is that the volume decrease results from the "unmasking" of charged groups as a consequence of the transition (Zurawski & Foster, 1974). Linderstrøm-Lang and Jacobsen (1941) calculated that the formation of an ion pair from an uncharged base–acid pair, e.g., an amine and carboxylate pair, would produce a ΔV of about −17 mL/mol of ion pair formed. Incidentally, these volume data argue against the view proposed by Harmsen et al. (1971) that the neutral transition involves the alteration of the pK of the imidazole groups in BSA, thereby causing a pH shift in serum albumin. To produce the magnitude of volume change observed would require amounts of histidyl residues substantially in excess to the number present in BSA.

A seeming divergence between these two systems occurs when $\nu \geq 3$; at pH 5.3 the values for $\partial(\Delta V)/\partial\nu$ increase sharply whereas at pH 7.4 this function exhibits a small decrease with an increase of binding. One recalls that $\sim$2 mol of protons are liberated per mol of Cu(II) bound (Gurd, 1970; Reynolds et al., 1973). At pH 5.3 the protons combine with the carboxylate residues, producing a sizable volume increase (Katz & Miller, 1971a); at the elevated pH the proton sink is provided by 23 histidyls in BSA, resulting in a small volume decrement (Katz & Miller, 1971b).

A reviewer suggested that the singular behavior of the Cu(II)–BSA system at pH 7.4 may be caused by the aggregation of the protein (Saroff & Choate, 1958; Österberg et al., 1975). However, copper-catalyzed polymerization is a slow time-dependent process involving disulfide interchange (Klotz et al., 1955). The volume changes reported here occurred immediately after mixing and were essentially invariant for a 120-min period.

The addition of Cu(II) to BSA at physiological pH induces a structural transition which is manifested by a cooperative binding isotherm and apparently anomalous volume effects. The cooperative binding isotherm produced by Cu(II) with BSA at physiological pH may constitute a protective or control device for regulating cupric ion concentration in plasma, i.e., if the plasma cupric ion concentration falls below the physiological norm, then Cu(II) will be released from albumin; however, at elevated Cu(II) concentrations, albumin will be converted to the high-affinity form and will reduce the cation concentration to an acceptable level (Figure 2). It will be of interest to establish whether other divalent cations can induce this transition in view of similar pH dependence exhibited by calcium ion (Katz & Klotz, 1953; Harmsen et al., 1971).

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