5

STOICHIOMETRY, KINETICS, AND THERMODYNAMICS OF CALCIUM BINDING

One wants to know several fundamental characteristics about the binding of calcium, or of any ligand, to a protein:

1. Stoichiometry, affinity, and cooperativity of binding
2. Kinetics of binding
3. Partition of free energy of binding (ΔG) among enthalpy (ΔH) and entropy (ΔS)
4. Changes in structure associated with binding
5. Changes in function associated with binding

In this chapter we develop the theory and formalism of stoichiometry, affinity, cooperativity, kinetics, and thermodynamics. Chapter 6 covers the techniques used to measure calcium binding. Complexes of proteins with metals other than calcium are surveyed in Chapters 8 and 9. In Chapters 10 to 15 we characterize the basic structures, characteristics, and functions of the many homolog families of calcium binding proteins.

5.1. STOICHIOMETRY, AFFINITY, AND COOPERATIVITY OF BINDING

Two fundamental characteristics of any metal (ligand) binding protein are the number of metal ions bound (stoichiometry) and the affinity(s) of binding.

Calcium Binding Proteins, By Eugene A. Permyakov and Robert H. Kretsinger

Consider the reaction

$$\mathrm{P} + \mathrm{Me} \leftrightarrow \mathrm{PMe} \tag{5.1}$$

in which P is a protein and Me is a metal ion. If one can determine experimentally any three of the following five concentrations: free protein, [P]; or [Me]; or [PMe]; or $[P_0]$ (total protein concentration, $[P] + [PMe]$); or $[Me_0]$ (total metal ion concentration, $[Me] + [PMe]$) (excepting the two triplets [P], [PMe], and $[P_o]$ or [Me], [PMe], and $[Me_o]$), one can compute the dissociation constant:

$$K_d = \frac{[P][Me]}{[PMe]} (\text{molar}) \tag{5.2}$$

$$\nu = \frac{[PMe]}{[P_0]} = \frac{[Me]}{K_d + [Me]} \tag{5.3}$$

In the case of n independent, equivalent sites, equation (5.3) becomes

$$\nu = \frac{[PMe]}{[P_0]} = \frac{n[Me]}{K_d + [Me]} \tag{5.4}$$

or

$$\frac{\nu}{[Me]} = \frac{n}{K_d} - \frac{\nu}{K_d} \tag{5.5}$$

This is the well-known *Scatchard representation*. The plot of $\nu/[Me]$ against ν is called the *Scatchard plot*. The slope of the straight line of the Scatchard plot gives K_d, and the point of its intersection with the ordinate axis gives n. In the case of several independent binding sites with dissociation constants K_{di},

$$\nu = \sum \frac{n_i[Me]}{K_{di} + [Me]} \tag{5.6}$$

Ideally, one should measure activities, not concentrations; however, the difference between concentration and activity is usually within the experimental error of the measurements for these systems.

The change of apparent standard free energy upon the binding of the ith molecule of the ligand is

$$\Delta G_i^0 = \mathrm{RT} \ln K_{di} \tag{5.7}$$

where R is the gas constant and T is the absolute temperature. Since

$$\Delta G_i^0 = \Delta H_i^0 - T \Delta S_i^0 \tag{5.8}$$

where ΔH_i^0 and ΔS_i^0 are standard enthalpy and entropy change, respectively. It follows that

$$\ln K_{di} = -\frac{\Delta H_i^0}{RT} + \frac{\Delta S_i^0}{R} \tag{5.9}$$

Differentiation of this expression yields the *van't Hoff equation:*

$$\frac{d \ln K_{di}}{dT} = \frac{\Delta H_i^0}{RT^2} \tag{5.10}$$

The values of calcium dissociation constants for various calcium binding proteins are within the range 10^{-9} to 10^{-4} M, depending on the type of the binding site. Intracellular calcium binding proteins (e.g., calmodulin, troponin C, parvalbumin) have the highest affinities for Ca^{2+} ions. Usually, their dissociation constants are comparable with intracellular calcium concentrations. The same generalization obtains for dissociation constants for magnesium; their values are comparable with intracellular concentrations of free Mg^{2+} ion. This same correspondence of concentration and affinity also obtains for sodium and potassium binding to these proteins. This means that several metal cations that occur in biological systems, the most important of which is magnesium, compete with calcium for well defined calcium binding sites. These balances of protein affinities and abundances of intracellur calcium and magnesium reflect the results of natural selection, not the affinities of small molecule ligands.

As noted in Chapters 3 and 4, the affinities of magnesium for many small, oxygen containing ligands exceeds that of calcium. In contrast, for most proteins, especially those in the EF-hand family, the affinity for calcium is three to five orders of magnitude higher than that for magnesium. This reflects the difficulty of wrapping a protein polymer around the Mg^{2+} ion in nearly rigid octahedral coordination relative to the more flexible pentagonal bipyramid of oxygens surrounding the Ca^{2+} ion and also the higher energy of interaction of Mg^{2+} ions with water molecules compared with Ca^{2+} ions.

If the protein has several interacting binding sites, the situation is complicated and the experimental challenge is more difficult. For n cooperative equivalent binding sites:

$$\mathrm{P} + n\mathrm{Me} \leftrightarrow \mathrm{PMe}_n \tag{5.11}$$

$$K_d = \frac{[\mathrm{P}][\mathrm{Me}]^n}{[\mathrm{PMe}_n]} \quad (\mathrm{molar}^n) \tag{5.12}$$

$$\frac{\nu}{n} = \frac{[\mathrm{Me}]^n}{K_d^n + [\mathrm{Me}]^n} \tag{5.13}$$

In practice there is never absolute cooperativity, and experimental data reflecting cooperative binding are usually described by equations (5.12) and (5.13) with

Hill coefficient n_h instead of n: $1 \leq n_h \leq n$ in the case of positive cooperativity. When $n_h = n$ the system behaves fully cooperatively; when $n_h = 1$ there is no cooperativity. In negative cooperativity the binding of one Me reduces the affinity of the protein for subsequent Me's; n_h is less than 1.

Several complexities are encountered in practice. As discussed in Chapters 10 to 15, most calcium binding proteins have more than one calcium binding site. Although there is evidence of some cooperativity among these multiple sites, whatever cooperativity exists might vary for binding different cations. To the extent that cooperativity exists, there is not yet evidence of its physiological function.

Of greater concern, other cations might bind to sites on the protein that differ from calcium binding site(s). Such binding to an alternative site(s) might affect the affinity for calcium at its major site(s). These alternative situations are difficult to sort out, especially without knowledge of their various structures.

There are a few proteins with a single strong calcium binding site, such as α-lactalbumin, found in milk and as a component of lactose synthase. It is a favorite subject for evaluation of techniques and concepts.

There are many proteins with two strong calcium binding sites: for example, parvalbumins, calbindin 9_{kD}, and recoverin. The main difficulties in studying even two site calcium binding proteins are elucidation of the mechanism of the binding and determining whether the binding of one Ca^{2+} ion increases (positive cooperativity) or decreases (negative cooperativity) the affinity for calcium at the second site. In practice, it is difficult to determine the binding mechanism, even in the case of two binding sites. Consider the binding of Me to a protein P with two binding sites:

$$\begin{array}{ccc} & \overset{K_{11}}{} & \\ \mathrm{P} & \leftrightarrow & \mathrm{MeP} \\ \updownarrow K_{12} & & \updownarrow K_{22} \\ \mathrm{PMe} & \leftrightarrow & \mathrm{MePMe} \\ & \underset{K_{21}}{} & \end{array} \tag{5.14}$$

where K_{11}, K_{22}, K_{12}, and K_{21} are equilibrium dissociation constants for the two binding sites:

$$K_{11} = \frac{[\mathrm{P}][\mathrm{Me}]}{[\mathrm{MeP}]} \tag{5.15}$$

$$K_{22} = \frac{[\mathrm{MeP}][\mathrm{Me}]}{[\mathrm{MePMe}]} \tag{5.16}$$

$$K_{12} = \frac{[\mathrm{P}][\mathrm{Me}]}{[\mathrm{PMe}]} \tag{5.17}$$

$$K_{21} = \frac{[\mathrm{PMe}][\mathrm{Me}]}{[\mathrm{MePMe}]} \tag{5.18}$$

Concentrations of the reactants are related to each other by the equations of mass balance:

$$[P] + [PMe] + [MeP] + [MePMe] = [P_0] \tag{5.19}$$

$$[Me] + [PMe] + [MeP] + 2[MePMe] = [Me_0] \tag{5.20}$$

where $[P_0]$ and $[Me_0]$ are total protein and total metal concentrations.

The equilibrium binding constants are related by

$$K_{22}K_{21} = K_{12}K_{11} \tag{5.21}$$

$$\text{If} \quad K_{11} = K_{21}, \quad \text{then} \quad K_{12} = K_{22} \tag{5.22}$$

This means that the two calcium binding sites are independent [equation (5.22)] and the binding in each site occurs independent of the other site. One can then simplify:

$$K_{11} = K_{21} = K_1 \tag{5.23}$$

$$K_{12} = K_{22} = K_2 \tag{5.24}$$

If $K_2 \gg K_1$, the first site is filled (almost) completely before the second begins to fill. Scheme (5.14) simplifies to the sequential binding scheme

$$\mathrm{P} \overset{K_1}{\longleftrightarrow} \mathrm{MeP} \overset{K_2}{\longleftrightarrow} \mathrm{MePMe} \tag{5.25}$$

If K_{11} is similar to K_{12}, then K_{21} must be similar to K_{22}; one measures the apparent dissociation constants:

$$\frac{1}{K_{1\mathrm{app}}} = \frac{[MeP] + [PMe]}{[P][Me]} = \frac{1}{K_{11}} + \frac{1}{K_{12}} \tag{5.26}$$

$$\frac{1}{K_{2\mathrm{app}}} = \frac{[MePMe]}{([MeP] + [PMe])[Me]} = \frac{1}{K_{11} + K_{12}} \tag{5.27}$$

It is difficult to distinguish experimentally between schemes (5.14) ($K_{11} \neq K_{12} \neq K_{21} \neq K_{22}$) and (5.22) ($K_{11} = K_{21} = K_1$ and $K_{12} = K_{22} = K_2$) as well as between schemes (5.22) and (5.25) ($K_2 \gg K_1$).

Consider a protein P binding a compound Me with dissociation constant K_d. In the simplest case, one P binds one Me, as described previously:

$$P + Me \leftrightarrow PMe \tag{5.1}$$

$$K_d = \frac{[\mathrm{P}][\mathrm{Me}]}{[\mathrm{PMe}]} \tag{5.28}$$

$$[\mathrm{P}] + [\mathrm{PMe}] = [\mathrm{P_0}] \tag{5.29}$$

$$[\mathrm{P}] = \frac{[\mathrm{P_0}]K_d}{K_d + [\mathrm{Me}]} \tag{5.30}$$

$$[\mathrm{PMe}] = \frac{[\mathrm{P_0}][\mathrm{Me}]}{K_d + [\mathrm{Me}]} \tag{5.31}$$

Combining equations, one obtains

$$[\mathrm{Me}]^2 + \{K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])\}[\mathrm{Me}] - [\mathrm{Me_0}]K_d = 0 \tag{5.32}$$

The solution of this quadratic equation is

$$[\mathrm{Me}] = \left\{ \frac{[K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])]^2}{4 + [\mathrm{Me_0}]K_d} \right\}^{1/2} - \frac{K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])}{2} \tag{5.33}$$

Usually, to study the binding of Me to a protein by physical methods such as fluorescence spectroscopy, circular dichroism spectroscopy, nuclear magnetic resonance, and so on, the protein solution is titrated by small additions of Me, and physical parameters (e.g., fluorescence parameters of the protein) are measured after each addition. Fluorescence quantum yield (or fluorescence intensity at a fixed wavelength, Chapter 6) changes following the equation

$$q = \frac{q_{\mathrm{P}}[\mathrm{P}]}{[\mathrm{P_0}]} + \frac{q_{\mathrm{PMe}}[\mathrm{PMe}]}{[\mathrm{P_0}]} \tag{5.34}$$

in which q_{P} and q_{PMe} are fluorescence quantum yields of P and PMe, respectively. The theoretical dependence of q on concentration $[\mathrm{Me_0}]$ is

$$q = \frac{\begin{array}{c} 2q_{\mathrm{P}}K_d + q_{\mathrm{PMe}}(\{[K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])]^2/4 + [\mathrm{Me_0}]K_d\}^{1/2} \\ -[K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])]/2) \end{array}}{2K_d + \{[K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])]^2 + 4K_d[\mathrm{Me_0}]\}^{1/2} - [K_d + ([\mathrm{P_0}] - [\mathrm{Me_0}])]} \tag{5.35}$$

The shape of the curve $q([\mathrm{Me_0}])$ depends on the relationship between $[\mathrm{P_0}]$ and K_d (Figure 5.1). If $K_d \ll [\mathrm{P_0}]$, the dependence $q([\mathrm{Me_0}])$ is a straight line with an abrupt break (saturation) at $[\mathrm{Me_0}] = [\mathrm{P_0}]$. If $K_d = [\mathrm{P_0}]$, the curve $q([\mathrm{Me_0}])$ is far from saturation at $[\mathrm{Me_0}] = [\mathrm{P_0}]$. If $K_d \gg [\mathrm{P_0}]$, the curve is even less steep. The same relationships are valid in the more complicated case of a multisite protein.

It is evident that if $[\mathrm{P_0}] \gg K_d$, it is impossible to determine the binding constant from the $q([\mathrm{Me_0}])$ curve, but the stoichiometry of binding is seen clearly in this case. In contrast, if $[\mathrm{P_0}] \ll K_d$, it is difficult to determine stoichiometry (the

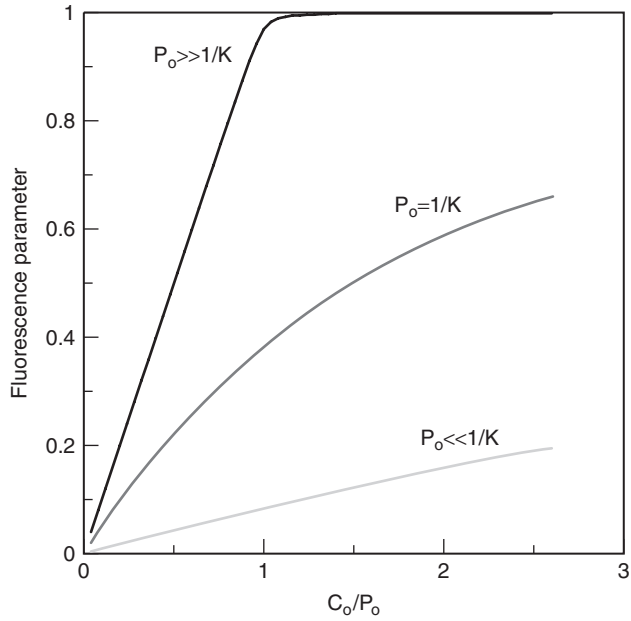

Figure 5.1. Model curves of spectrofluorimetric titration of a single-site protein by metal ions at different ratios of protein concentration and dissociation constant of the complex.

curve is very smooth), but the binding constant can be measured with sufficient accuracy. In the case of a very low K_d value, it is usually impossible to decrease $[P_0]$ so that it would be comparable with K_d because of the limited sensitivity of the physical instrument (e.g., spectrofluorimeter). One is then forced to carry out measurements under the conditions in which $[P_0] \gg K_d$. This does not permit determination of the binding constant.

In the opposite extreme, of a very high value of K_d, it is easy to measure its value if the binding stoichiometry is known. In this case the curve $q([Me_0])$ plateaus at very high $[Me_0]$ concentrations when $[Me_0] \gg K_d$ and the concentration of free [Me] is approximately equal to $[Me_0]$.

As mentioned above, the optimal situation is when $[P_0]$ is comparable to K_d. In this case one can measure both the stoichiometry and the binding constant. A convenient method of evaluation of K_d is computer fitting of the theoretical curve $q([Me_0])$ to experimental points by means of variation of K_d. Even in the absence of any other components in solution except for the protein and Ca^{2+} ions, complete characterization of the calcium binding properties of a protein is a challenging task.

5.2. KINETICS OF BINDING

It is important to know the association (k_{on}, M^{-1} s^{-1}) and dissociation (k_{off}, s^{-1}) rate constants when studying cell signaling. These on and off rates are related to

the equilibrium constant by

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}} \tag{5.36}$$

For the stronger calcium binding sites, k_{on} is often very close to the diffusion-controlled limit, about 10^9 M^{-1} s^{-1}. For Mg^{2+} ions, k_{on} is usually several orders of magnitude lower than that for Ca^{2+} ions, while the k_{off} values for calcium and magnesium are close to one other.

5.3. PARTITION OF FREE ENERGY OF BINDING (ΔG) AMONG ENTHALPY (ΔH) AND ENTROPY (ΔS)

The thermal unfolding of most calcium binding proteins does not lead to the formation of a random coil structure (the main-chain [φ, ψ] and side-chain [χ's] dihedral angles are random, constrained only by van der Waals contacts). Instead, it results in the formation of a partially unfolded state with partially conserved metal binding sites. The affinity of this partially unfolded state(s) for calcium can be rather high and should be taken into consideration when evaluating in vitro experiments.

The situation may be additionally complicated since in the absence of bound cations, some metal binding proteins (e.g., α-lactalbumin and equine lysozyme) unfold at 20°C or even lower (Figure 5.2). In this case, the measurement of metal binding at room temperature gives only apparent metal binding constants and involves at least one intermediate state in the course of the measurements. Thus, the measurement of metal binding constants at a single fixed temperature may

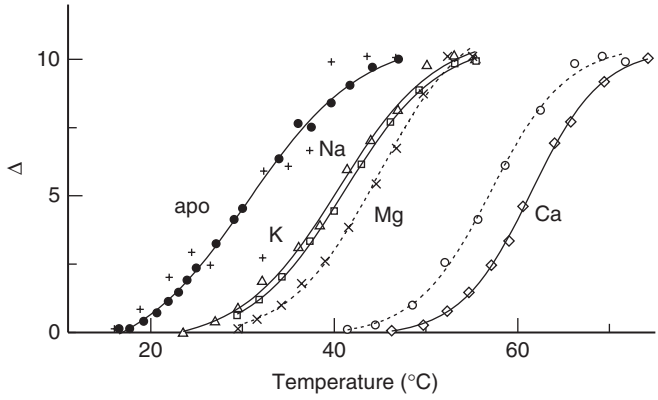

Figure 5.2. Thermal unfolding transition for various α-lactalbumin states (apo, Na^+-, K^+-, Mg^{2+}-, and Ca^{2+}-loaded) measured by intrinsic fluorescence. Δ is a fraction of conversion from the native to the thermally unfolded state.

lead to erroneous conclusions. To characterize the system completely, one should study metal binding at different temperatures and measure thermal unfolding of the protein in the presence of various metal concentrations. Given these data, one can construct a *phase diagram* using coordinates of free metal ion concentration and temperature (Permyakov and Permyakov, 2006).

Strictly speaking, the metal binding–induced structural or thermal transition in a protein cannot be considered as a classical phase transition, since certain distinctive features of phase transitions, such as the presence of a phase boundary, are absent in this case. Here, the term *phase transition* is applied to intramolecular transitions occurring within the protein molecule in response to metal association/dissociation or change of temperature. Nevertheless, in this case the phase diagram represents the most general characterization of the protein–metal system. It allows the easy visualization of regions of predominance of different protein states and the prediction of protein states under various experimental conditions. The phase diagram can be constructed relatively easily for proteins that possess a single metal binding site.

The equilibrium scheme of the binding of one metal ion (Me) to the protein molecule, taking into consideration equilibrium between native (P, PMe) and thermally changed (P*, P* Me) states of the protein, is

$$\begin{array}{ccc} & \overset{K_{\mathrm{Me}}}{} & \\ \mathrm{P} + \mathrm{Me} & \leftrightarrow & \mathrm{PMe} \\ \gamma \quad \Downarrow\Uparrow & & \Downarrow\Uparrow \quad \beta \\ \mathrm{P}^* + \mathrm{Me} & \leftrightarrow & \mathrm{P}^*\mathrm{Me} \\ & \underset{K^*_{\mathrm{Me}}}{} & \end{array} \tag{5.37}$$

in which K_{Me} and K^*_{Me} are intrinsic metal ion dissociation constants for the native and thermally denatured protein, respectively, and γ and β are equilibrium constants of the thermal denaturation of the protein in its apo and metal ion-bound forms, respectively:

$$K_{\mathrm{Me}} = \exp\left[\frac{(\Delta H_{\mathrm{Me}} - T\Delta S_{\mathrm{Me}})}{RT}\right] \tag{5.37}$$

$$K^*_{\mathrm{Me}} = \exp\left[\frac{(\Delta H^*_{\mathrm{Me}} - T\Delta S^*_{\mathrm{Me}})}{RT}\right] \tag{5.38}$$

$$\gamma = \exp\left[-\frac{(\Delta H_{\alpha} - T\Delta S_{\alpha})}{RT}\right] \tag{5.39}$$

$$\beta = \exp\left[-\frac{(\Delta H_{\alpha} - T\Delta S_{\beta})}{RT}\right] \tag{5.40}$$

ΔH_{Me}, ΔH^*_{Me} and ΔS_{Me}, ΔS^*_{Me} are enthalpy and entropy changes for the metal ion binding to native and thermally denatured protein. ΔH_{α}, ΔH_{β} and ΔS_{α}, ΔS_{β} are enthalpy and entropy changes for the thermal transitions in the apo and metal ion-bound protein. ΔH_{α} and ΔS_{β} can be determined using experiments

studying the thermal denaturation of the apo-protein, while ΔH_β and ΔS_β can be determined from the thermal denaturation curve for the metal ion-bound protein.

The apparent metal ion dissociation constant determined from the fluorescence experiment is

$$\frac{1}{K_{app}} = \frac{[PMe] + [P^*Me]}{([P] + [P^*])[Me]} = \frac{1}{K_{Me}} \frac{1+\beta}{1+\gamma} \tag{5.41}$$

$$\frac{K^*_{Me}}{K_{Me}} = \frac{\gamma}{\beta} \tag{5.42}$$

Knowledge of all the thermodynamic parameters for metal cation binding allows calculation of the thermal denaturation curve of the protein in the presence of any given concentrations of the metal ion. More important, this knowledge allows computation of a phase diagram using the free calcium–temperature coordinates. Figure 5.3 shows such phase diagrams for bovine α-lactalbumin and equine lysozyme, proteins with a single strong calcium binding site (Permyakov et al., 2006). The phase diagram depicts both regions of predominance of separate protein states and the areas of transition between them, including lines of half-transitions in temperature (1) and calcium (2) scales. Thus, knowing the current temperature and pCa ($-\log[Ca^{2+}]$) values, the actual protein state can be assessed. Moreover, limits of curves 1 and 2 obviously demonstrate midtransition

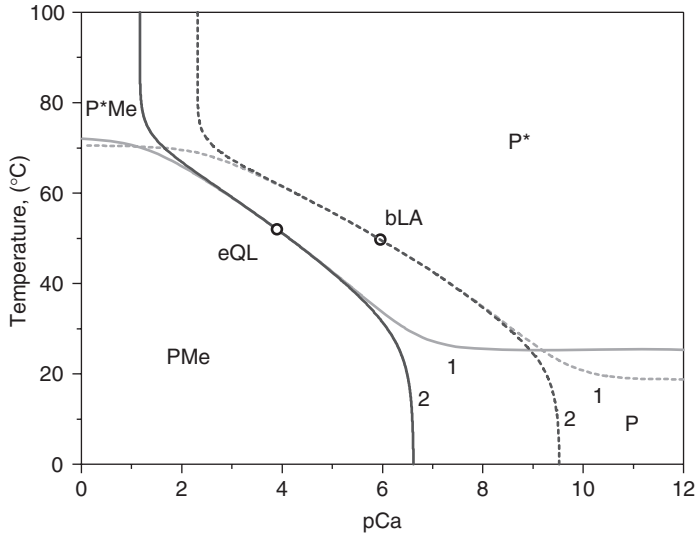

Figure 5.3. Phase diagram of equine lysozyme (eQL) and bovine α-lactalbumin (bLA) in the free calcium concentration–temperature space, calculated according to the four-states scheme (5.37). Curves 1 and 2 correspond to a half-transition for binding of calcium and thermal denaturation, respectively.

temperatures for apo- and calci-protein (curve 1, ordinate values) and calcium binding affinities of native and denatured states of protein (curve 2, abscissa values).

One is heartened by the expansion to proteins and improvement in techniques used to study calcium binding (Chapter 6). A great deal of empirical knowledge has been gathered, and the physical properties of these proteins have been related to their functions. Nonetheless, it is sobering to realize that given the sequence or even the structure of a protein, we cannot identify, with certainty, the site at which calcium might bind, and even if we can, we cannot estimate the binding affinity. Our ability to predict is poor; more disturbing, we do not really understand why.