Preface

Calcium plays an important role in a wide variety of biological processes. This divalent metal ion can bind to a large number of proteins; by doing so it modifies their biological activity or their stability. Because of its distinct chemical properties calcium is uniquely suited to act as an on–off switch or as a light dimmer of biological activities. The two books entitled *Calcium-Binding Protein Protocols* (Volumes I and II) focus on modern experimental analyses and methodologies for the study of calcium-binding proteins. Both extracellular and intracellular calcium-binding proteins are discussed in detail. However, proteins involved in calcium handling (e.g., calcium pumps and calcium channels), fall outside of the scope of these two volumes. Also, calcium-binding proteins involved in bone deposition will not be discussed, as this specific topic has been addressed previously. The focus of these two books is on studies of the calcium-binding proteins and their behavior in vitro and in vivo. The primary emphasis is on protein chemistry and biophysical methods. Many of the methods described will also be applicable to proteins that do not bind calcium.

Calcium-Binding Protein Protocols is divided into three main sections. The section entitled *Introduction and Reviews* provides information on the role of calcium in intracellular secondary messenger activation mechanisms. Moreover, unique aspects of calcium chemistry and the utilization of calcium in dairy proteins, as well as calcium-binding proteins involved in blood clotting, are addressed. The second section entitled *Calcium-Binding Proteins: Case Studies* provides a wealth of information about protein purification and characterization strategies, X-ray crystallography, and other studies that are focused on specific calcium-binding proteins. Together, these two sections comprise Volume I of this series. By introducing the various classes of intra- and extracellular calcium-binding proteins and their modes of action, these two sections set the stage and provide the necessary background for the third section. The final section entitled *Methods and Techniques to Study Calcium-Binding Proteins* makes up Volume II of *Calcium-Binding Protein Protocols.* Here the focus is on the use of a range of modern experimental techniques that can be employed to study the solution structure, stability, dynamics, calcium-binding properties, and biological activity of calcium-binding proteins in general. As well, studies of their ligand-binding properties and their distribution in cells are included. In addition to enzymatic assays and more routine spectroscopic and protein chemistry techniques, particular attention has been paid in the second volume to modern NMR approaches, thermodynamic analyses, kinetic mea-

surements such as surface plasmon resonance, strategies for amino acid sequence alignments, as well as fluorescence methods to study the distribution of calcium and calcium-binding proteins in cells. In preparing their chapters, all the authors have attempted to share the little secrets that are required to successfully apply these methods to related proteins. Together the two volumes of *Calcium-Binding Protein Protocols* provide the reader with a host of experimental methods that can be applied either to uncover new aspects of earlier characterized calcium-binding proteins or to study newly discovered proteins.

As more and more calcium-binding proteins are being uncovered through genome sequencing efforts and protein interaction studies (e.g., affinity chromatography, crosslinking, or yeast two-hybrid systems) the time seemed right to collect all the methods used to characterize these proteins in a book. The methods detailed here should provide the reader with the essential tools for their analysis in terms of structure, dynamics, and function. The hope is that these two volumes will contribute to our understanding of the part of the proteome, which relies on interactions with calcium to carry out its functions.

In closing, I would like to thank Margaret Tew for her invaluable assistance with the editing and organization of these two books. Finally, I would like to thank the authors of the individual chapters, who are all experts in this field, for their cooperation in producing these two volumes in a timely fashion.

Hans J. Vogel, PhD

2

Calcium Binding to Proteins Studied via Competition with Chromophoric Chelators

Sara Linse

1. Introduction

Optical spectroscopic techniques can be used to measure Ca^{2+}-binding constants when the Ca^{2+}-bound and free forms of the protein display a difference in, for example, the UV absorbance, CD or fluorescence spectrum, or fluorescence polarization. One may then start with the Ca^{2+}-free form, titrate in Ca^{2+} stepwise, measure a spectrum or intensity at each step, and obtain the binding constants from computer fitting to the data. The best accuracy is achieved when the protein concentration is roughly the same as the dissociation constant (the inverse of the binding constant) such that there are significant populations of both bound and free forms at several titration points. This limits the useful range of such direct measurements to binding constants below $10^6\ M^{-1}$ (K_D > 1 μ*M*), because of the practical difficulty of making buffers with less than 0.5–1 μ*M* free Ca^{2+}. For Ca^{2+}-binding proteins with affinities of $10^6\ M^{-1}$ and up, one has to rely on indirect measurements. One popular such approach uses around 1 m*M* ethylenediaminetetracetic acid (EDTA) or ethylene glycol-*bis* *N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), and a much smaller amount of protein so that the free-Ca^{2+} concentration is essentially controlled by the Ca^{2+}-buffering capacity of EDTA or EGTA. A potential risk with such approaches is binding of EDTA or EGTA to the protein with consequences for its Ca^{2+} affinity. Another type of indirect approach outlined in this chapter involves the use of a chelator whose absorbance or fluorescence is Ca^{2+} dependent ***(1–3)***. A mixture of equal (10–50 μ*M*) amounts of chelator and protein is titrated with Ca^{2+} and the binding to the chelator is monitored spectroscopically. The Ca^{2+}-binding constants of the protein are extracted by fitting to the absorbance or fluores-

From: *Methods in Molecular Biology*, vol. 173:
Calcium-Binding Protein Protocols, Vol. 2: Methods and Techniques
Edited by: H. J. Vogel

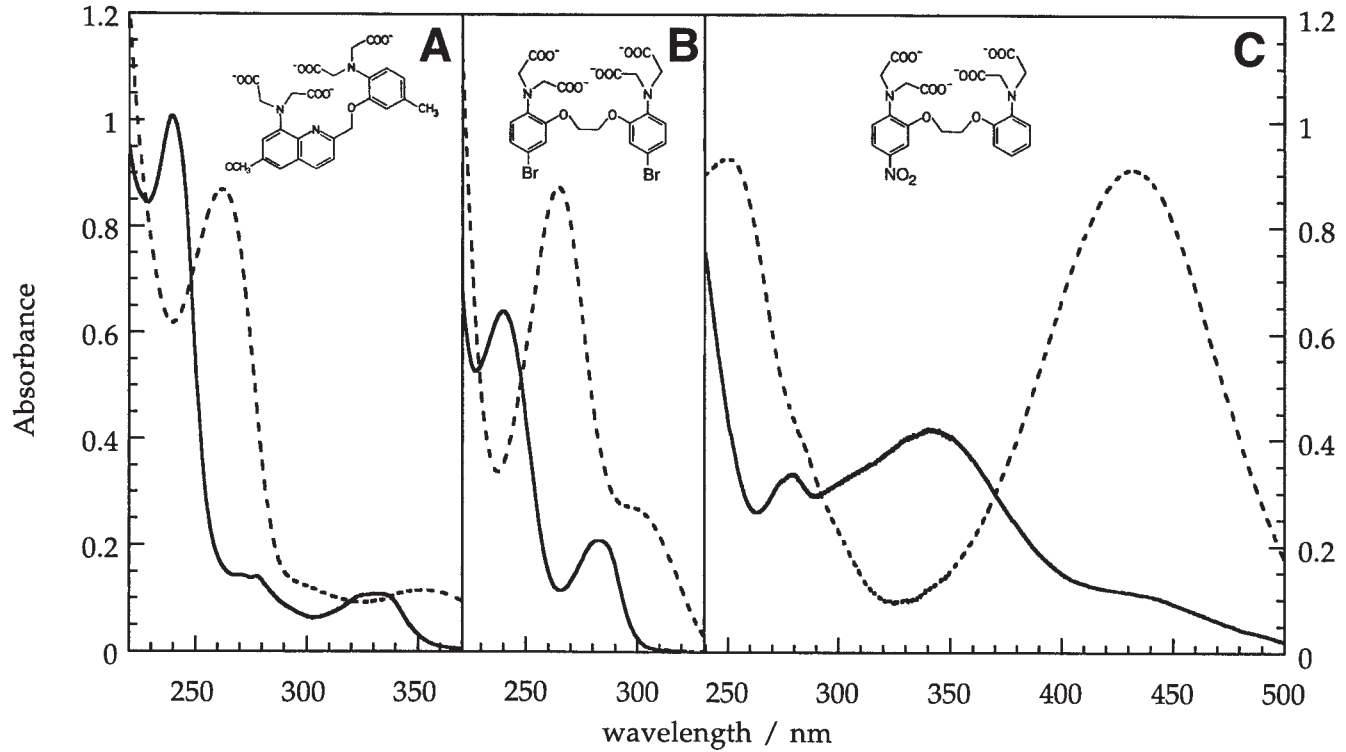

Fig. 1. Molecular structures and absorbance spectra of **(A)** quin-2; **(B)** 5,5'Br_2-BAPTA; and **(C)** 5N-BAPTA. (- - -) calcium free and (—) calcium bound forms.

Table 1
Molecular Structures, Spectra and Properties of 3 Chelators

Chelator	λ_{max}/nm	ε/M/cm	*KD*/M[a] low salt[b]	*KD*/*M* 0.15 *M* KCl	*KD*/*M* 0.15 *M* NaCl	*Mw*	ref.
quin-2	239.5	$4.2 \cdot 10^4$	$5.2 \cdot 10^{-9}$	$1.2 \cdot 10^{-7}$		694[c]	***1,2***
5,5'Br_2-BAPTA	239.5	$1.4 \cdot 10^4$	$1.0 \cdot 10^{-7}$	$2.3 \cdot 10^{-6}$	$1.4 \cdot 10^{-6}$	787[c]	***1–4***
5N-BAPTA	340	$6.0 \cdot 10^3$	$1.7 \cdot 10^{-6}$		$2.7 \cdot 10^{-5}$	521[d]	***1,4***

[a]All KD's are in 2 m*M* Tris-HCl at pH 7.5.
[b]No salt added beyond the HCl needed to set the pH.
[c]Tetra potassium salt.
[d]Free acid.
Quin-2 can be obtained from Fluka, Buchs, Switzerland, and 5,5'Br_2-BAPTA and 5N-BAPTA from Molecular Probes, Eugene, OR.

cence as a function of total Ca^{2+} concentration. This method gives very high precision in the deduced constants, but the accuracy is never better than the accuracy in the Ca^{2+} affinity for the chelator. Although much lower concentrations of chelator are used, this method is also potentially hampered by interactions between chelator and protein. Another source of errors are electrostatic screening effects from highly charged proteins that perturb the calcium affinity for the chelator from its value in a protein-free solution.

2. Materials

1. UV absorbance or fluorescence spectrometer.
2. Quartz cuvets.
3. Chromophoric calcium chelator. An ideal chelator is one with a calcium affinity close to that of the protein to be studied. This will ensure that the calcium ions are roughly evenly distributed between the chelator and protein leading to high precision in the binding constants for the protein. The molecular structures, spectra and properties of three useful chelators are summarized in **Fig. 1** and **Table 1**.
4. Ca^{2+}-free buffer (*see* **Note 1**). To get the buffer Ca^{2+} free, prepare in double-distilled water (ddH_2O) in a plastic container and put a dialysis tube filled with Chelex-100 resin (Bio-Rad) in the container before adjusting the pH (*see* **Note 2**). Before use, the dialysis tube has to be boiled four times in ddH_2O and the chelex has to be neutralized and washed with ddH_2O. Let the buffer rest for a few days before use to reduce free Ca^{2+}.
5. 3 m*M* $CaCl_2$. Weigh as accurately as you can 44.106 mg $CaCl_2 \cdot 2H_2O$ (*see* **Note 3**). Note the exact weight and calculate the Ca^{2+} concentration from that value. Dissolve the Ca^{2+}-free buffer in a 100-mL volumetric flask. Adjust the pH, if necessary, and fill up the flask. Aliquot into a large number of Eppendorf tubes and freeze the tubes. For each titration, use one tube and then dispose.
6. 1 *M* $CaCl_2$. Dissolve 14.72 g $CaCl_2 \cdot 2H_2O$ in 100 mL ddH_2O and adjust pH to 7.5.

7. 0.1 *M* EDTA. Dissolve 37.22 g EDTA in 100 mL ddH_2O. Add concentrated NaOH to get the EDTA into solution and adjust the pH to 7.5.
8. 5 m*M* EDTA. Dilute 25 mL 0.1 *M* EDTA with 475 mL ddH_2O in a squeeze bottle.

3. Method

3.1. Experimental Procedure

1. A Ca^{2+}-free solution of 25–30 μ*M* chelator is prepared in the Ca^{2+}-free buffer. The exact chelator concentration C_Q is determined by withdrawing 2.5 mL, adding 5 μL 1 *M* $CaCl_2$ and recording the absorbance at λ_{max} (*see* **Table 1**). The chelator concentration is calculated as $CQ = A_{\lambda max}/\varepsilon$. The value of ε at λ_{max} is found in **Table 1**.
2. Rinse the cuvet once with ddH_2O. Fill with 5 m*M* EDTA and let sit for 1 min. Rinse several times with ddH_2O and finally with ethanol and dry the cuvet with nitrogen gas.
3. Record the absorbance at 263 nm (*see* **Note 4**) A_{263} for 2.5 mL of the chelator solution (-> A_1). Add 5 μL 0.1 *M* EDTA and record A_{263} (-> A_2). Add 5 μL 1 *M* $CaCl_2$ and record A_{263} (-> A_3). The calcium concentration in the chelator solution Ca_Q can be estimated as

$$Ca_Q = C_Q \cdot (A_2 - A_1) / (A_2 - A_3) \quad (1)$$

   Ideally, this value is below 1 μ*M* (*see* **Note 5**).
4. Rinse the cuvet once with ddH_2O. Fill with 5 m*M* EDTA and let sit for 1 min. Rinse several times with ddH_2O and, finally, with ethanol, and dry the cuvet with nitrogen gas.
5. Dissolve lyophilized Ca^{2+}-depleted protein (*see* **Note 6**) in the (Ca^{2+}- and EDTA-free) chelator solution to obtain a protein concentration of 25–30 μ*M*. This is the titrand, i.e., the solution that will be titrated with calcium.
6. Record A_{263} (*see* **Note 4**) for the titrand.
7. Add a Ca^{2+} aliquot (*see* **Note 7**) to the titrand and mix. Record A_{263} (*see* **Note 8**).
8. **Step 7** is repeated until no significant change has occurred in A_{263} over the last five points, beyond what would be caused by dilution (*see* **Note 9**).

3.2. Computer Fitting

The chelator method can be used to determine *macroscopic* Ca^{2+}-binding constants of a protein. Because the measured quantity contains no information about the distribution of calcium among separate sites in the protein, *microscopic* binding constants cannot be determined. The *macroscopic* binding constants K_1, K_2–K_N are defined as follows:

$$\begin{aligned} &P + Ca^{2+} \rightleftharpoons PCa && K_1 = [PCa]/([P][Ca^{2+}]) \\ &PCa + Ca^{2+} \rightleftharpoons PCa_2 && K_2 = [PCa_2]/([PCa][Ca^{2+}]) \\ &\ldots \\ &PCa_{N-1} + Ca^{2+} \rightleftharpoons PCa_N && K_N = [PCa_N]/([PCa_{N-1}][Ca^{2+}]) \end{aligned} \quad (2)$$

K_1–K_N (where N is the number of sites that are strong enough to compete with the chelator) are obtained by nonlinear least squares fitting to the absorbance as a function of total calcium concentration. An analysis based on concentration (not activities) can be performed as follows (*see* **Note 10**).

The total Ca^{2+}-concentration at each titration point i ($CATOT_i$), is calculated from the initial (*see* **Note 11**) and added Ca^{2+}. A nominal value for the protein concentration at each titration point (CP_i) is calculated from the initial protein concentration based on the weight of the lyophilized protein. $CATOT_i$ and CP_i are adjusted for the dilution imposed by the calcium additions, as is CQ_i, the chelator concentration at titration point i. Fixed parameters in the fit are KDQ, CQ_i, CP_i, and $CATOT_i$. KDQ is the Ca^{2+}-dissociation constant of the chelator. Variable parameters in the fit are K_1–K_N, AMAX, AMIN, and F. AMAX and AMIN are the absorbances that the initial (nondiluted) solution would have had if it was completely Ca^{2+}-free or contained saturating amounts of Ca^{2+}, respectively. F is a correction factor that accounts for the fact that the protein concentration obtained by weight can be off by 10–20% because of residual water in lyophilized protein and because of errors in weight caused by the small (0.7–1.5 mg) quantities used (*see* **Note 12**).

For each set of values of the variable parameters, the Newton-Raphson method is used to solve the free Ca^{2+} concentration, Y, at each titration point, i, from the following equation:

$$Y = CATOT_i - \frac{CQ_i \cdot Y}{Y + KDQ} - \frac{F \cdot CP_i \sum_{k=1}^{N} (kY_k \cdot \prod_{j=1}^{k} K_j)}{1 + \sum_{k=1}^{N} (Y_k \cdot \prod_{j=1}^{k} K_j)} \tag{3}$$

which states that the free Ca^{2+} equals the total Ca^{2+} subtracted by the chelator-bound Ca^{2+} and the protein-bound Ca^{2+}. The absorbance at point i is calculated as

$$Acalculated,i = \left[AMAX - (AMAX - AMIN) \cdot \frac{Y}{Y + KDQ} \right] \cdot \frac{CQi}{Q_1} \tag{4}$$

where CQ_1 is the initial chelator concentration. Thus the changes in absorbance are assumed to arise from the chelator only. The sum of the squares of residuals (or error square sum) χ^2, is obtained by summing over all points in the titration

$$\chi^2 = \sum (A_{calculated,i} - A_{measured,i})^2 \tag{5}$$

The variable parameters are iterated in a separate procedure until an optimal fit (minimum χ^2) is found. Start with initial guesses at both sides of the parameter values of best fit, to make sure that the same result is obtained. To estimate the errors in the parameter values, one may fix one parameter, for example K_1, and

iterate the other parameters to obtain an optimal fit. Then fix K_1 at a new value and fit again. Repeat until you have found the values of K_1 that lead to a doubling of χ^2. In general, AMAX, AMIN, and F are better determined than the binding constants (*see* **Note 13**). If the protein binds calcium with positive cooperativity (*see* **Note 14**), the product of the binding constants is better determined than the individual constants.

3.3. Stoichiometry of Calcium Binding

The chelator method can be used to measure the stoichiometry of calcium binding. For such applications, extra care has to be taken to measure the protein concentration of the titrand and its initial and final calcium concentration.

1. Dissolve the protein in 3 mL chelator solution to approx 30 μ*M*.
2. Withdraw 200 μL. Freeze dry for acid hydrolysis.
3. Use 2.5 mL as titrand.
4. Save the rest for atomic absorption spectroscopy for initial calcium concentration analysis.
5. Record A_{263} for the titrand.
6. Add a calcium aliquot to the titrand and mix. Record A_{263}.
7. Repeat **step 6** until no significant A_{263} change has been observed over the last five points.
8. Withdraw an aliquot of the titrated titrand for atomic absorption spectroscopy for calcium analysis.
9. In the computer fitting, set the initial protein concentration to the value obtained from the amino acid analysis, and use a fixed factor F = 1.0. The number of macroscopic binding constants needed to obtain an optimal fit will be the same as the number of sites with affinities of similar value as the chelator. The initial calcium concentration used in the fit is obtained from the analysis at **step 4**. Check that the total calcium concentration at the last titration point is equal to the value obtained from the analysis at **step 8**.

3.4. Examples of Titration Data

Examples of experimental data and fitted curves are shown in **Fig. 2**. In the absence of calcium binding to the protein, the absorbance will decrease linearly until the total calcium concentration equals the chelator concentration. A linear decrease will be seen also when the protein has a site with the same Ca^{2+} affinity as the chelator, but more calcium will be needed to saturate the chelator. If the protein binds calcium weaker or stronger than the chelator, the binding curve will be no longer be a straight line, but will bend in a different direction depending on whether the affinity for the protein is higher or lower than for the chelator (*see* **Fig. 2A**). Examples of experimental data for proteins with one, two, or three high-affinity calcium-binding sites are shown in **Fig. 2B**. When the protein binds calcium at more than one site in a sequential manner

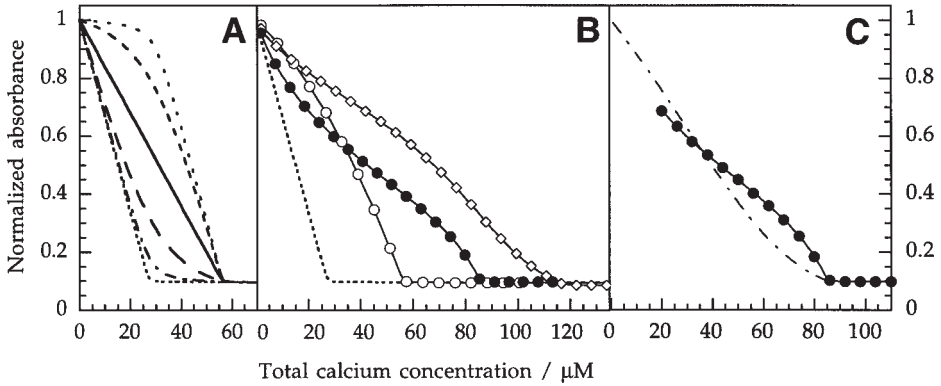

Fig. 2. The absorbance at 263 nm as a function of total calcium concentration for a mixture of 27.5 μ*M* quin-2 and 30 μ*M* protein. **(A)** Simulated curves for three proteins, each with one calcium-binding site with the same (—), 100-fold higher (short dashes), 10-fold higher (dashes), 10-fold lower (long dashes), or 100-fold lower (dash dotted) affinity than quin-2, plus one curve for chelator in the absence of protein (closely spaced short dashes). **(B)** Experimental data (symbols) and fitted curves (solid lines) for three proteins with different stoichiometries of calcium binding: (o) α-lactalbumin, lgK_1 = 8.7, (•) calbindin D_{9k} (recombinant bovine minor *A* with a P43M substitution), lgK_1 = 7.75. lgK_2 = 8.59 ***(5)***,(·) calerythrin lgK_1 = 8.08. lgK_2 = 9.10, lgK_3 = 7.57 ***(6)***. Simulated curve for chelator alone (dashed line). **(C)** (•) experimental data for calbindin D_{9k} contaminated with 20 μ*M* Ca^{2+} (the initial nondefined part of the fitted line is omitted). Simulated curve (dash dotted) for a protein with *lgK* = 7.5, contaminated with 30 μ*M* EDTA.

the titration curve may be S-shaped. Positive cooperativity (*see* **Note 14**) of Ca^{2+}-binding is also manifested as an S-shape in the titration curve as observed for calbindin D_{9k} (**Fig. 2B**,**C**), but the curvature is opposite to that of sequential binding. Calerythrin (**Fig. 2B**) first binds calcium to two sites with positive cooperativity, and then to a third weaker site, which is seen as two interlocked and opppositely bent Ss. As illustrated in **Fig. 2C**, a contamination with EDTA may be observed as an initial strong phase that may not fit with the protein concentration. A contamination with calcium leads to loss of data in the beginning of the curve (*see* **Fig. 2C** and **Note 15**).

4. Notes

1. Buffers and pH. Examples of useful buffers are 2 m*M* Tris-HCl, pH 7.5, for low ionic-strength measurements or 2–10 m*M* Tris-HCl, pH 7.5, with added salt for higher ionic-strength conditions. The choice of pH depends on the pK_a values of titrable groups on the chelator, as well as on the protein. It is best to use a pH significantly far from any pK_a-values, so that the binding constants are not sensi-

tive to small alterations in pH. For 2-[[2-[*bis*(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[*bis*-(carboxymethyl)-amino]quinoline (quin-2), the highest pK_a value is 6.36, and for 5,5'-dibromo-1,2-*bis*(O-amino-phenoxy)-ethane-*N*,*N*,*N*,'*N*'-tetraacetic acid (5,5'-Br2BAPTA) it is 5.6 ***(1)***.

2. The chelex tube may shift the pH of the buffer. The buffer may also slowly adjust after the pH has been changed by several units. It is often wise to avoid bringing the pH all the way to the goal. Instead, stop 0.5 pH units above or below (at the side from where you start). Do the final adjustment after a day or two. Your HCl or NaOH stock may contain some calcium so it is often best to wait up to a week before using the buffer.
3. Beware that calcium chloride is hygroscopic.
4. Choice of wavelength. The method is of course not limited to measurements at 263 nm. The ideal wavelength is one at which the calcium induced absorbance change for the chelator is as large as possible while the absorbance for the protein is calcium-independent. For the chelators quin-2 (*see* **Fig. 1A**) and 5,5'Br_2-BAPTA (*see* **Fig. 1B**), the absorbance at 263 nm decreases as a consequence of Ca^{2+}-binding. **Equation 4**, of course, pertains equally well to a case with increasing absorbance, e.g., another chelator and/or another wavelength. For 5N-BAPTA (*see* **Fig. 1C**) it is convenient to use 430 nm where the protein has no absorbance.
5. If the calcium concentration is not below 1 μ*M*, the buffer may need to rest for a few days to reduce free calcium, or maybe the chelator solution was contaminated with calcium by accident. Sometimes it seems as if solutions get calcium contaminated if you just look at them. Common sources of contamination are pipet tips, pH electrode, and glassware.
6. If the protein cannot be lyophilized, it may be added from a stock solution and the concentration of the chelator solution adjusted for the dilution. The use of a stock solution, however, necessitates the use of one extra container, e.g., Eppendorf tube, which may lead to calcium contamination. The safest procedure is to weigh out lyophilized protein in a cuvet that has been washed with 5 m*M* EDTA, multiple times with ddH_2O, finally, with ethanol, and then dried. One procedure to decalcify a high affinity Ca^{2+}-binding protein is described in volume 1, Chapter 10.
7. Ideally, all additions are equally large to get evenly spaced points. The aliquot has to be sufficiently small to get enough points in the titration for obtaining good precision in the binding constants. It is good to have the chelator covered by at least five points and each site in the protein by an additional five points, plus approx five points for the baseline after the binding is saturated. Aliquots of 3, 4, or 5 μL of 3 m*M* $CaCl_2$ are often ideal. If the measurements are performed at high salt and/or the protein or chelator binds calcium with lower affinity, one may need to add calcium from a stronger stock solution (e.g., 10 m*M*) at the end of the titration to get closer to saturation. Examples of curves for a lower affinity chelator (5N-BAPTA) and protein can be seen in **ref. *4***.
8. It may take time to reach equilibrium. One often has to make a compromise between the time it takes to obtain a stable recording and minimized photobleaching

of the chelator. Another problem with waiting too long is that the protein and/or chelator may start to absorb calcium form the cuvet. It is often best to wait 20–60 s until the initial quick changes in absorbance have settled and then note the recording. For a slowly equilibrating system, the cuvet may be put in darkness for equilibration for a few minutes at each titration point.

9. For example, if your absorbance is around 0.2 and you add 5-µL calcium aliquots to 2.5 mL titrand, the dilution will cause the absorbance to drop by 0.0004 at each addition.
10. An in-house computer program that performs the described analysis can be obtained from the author at Sara.Linse@Fkem2.LTH.SE.
11. The initial total calcium concentration in the titrand before any calcium additions are made can be determined by atomic absorption spectroscopy. Another way is to let the titration data specify the initial calcium concentration Ca_0 by fitting the data using several different values of Ca_0, and choosing the one that gives a value of (AMAX-AMIN) that agrees with A_2–A_3, (*see* **Subheading 3.1., step 3**).
12. It may be dangerous to use an adjustable factor *F* if the stoichiometry is not known or if the chelator and/or protein binds calcium with a lower affinity so that the curve does not have a sharp corner at the point of saturation. Using *F* to correct for protein concentration errors, however, works fine with a set of proteins with high affinity and known stoichiometry, because *F* is often better determined by the data than by any other method.
13. Such error bars do not include systematic errors because of false values of the calcium affinity for the chelator. Hence, they are a measure of precision rather than accuracy. Because the method is based on competition between the protein and a chelator, the precision is often high, and when the aim is to study the effects of mutations or other modifications, the method can be very useful and reliable.
14. The free energy of interaction between binding events at separate sites $\Delta\Delta G$ cannot be measured by the chelator method because this is a microscopic property. However, the macroscopic binding constants can be used to calculate a lower limit to $-\Delta\Delta G$. For a protein with two sites, this limiting value is $RT \ln (4K_2/K_1)$ and equal to the true cooperativity if the two sites have equal affinities. For a more thorough discussion of cooperativity and how it can be measured, *see* **refs. *5***, ***7***, and ***8***.
15. If the initial calcium concentration is not precisely known, or if it is too high (several µ*M*) the precision in the determined macroscopic-binding constants will be reduced. In such cases, it is especially difficult to quantitate the cooperativity, as points are missing in the beginning of the curve (*see* **Fig. 2C**). The initial curvature is not defined and the separation of the total affinity into individual macroscopic binding constants becomes uncertain.

Acknowledgments

Helpful comments on the manuscript by Eva Thulin, Tord Berggård, and Jonas Fast are gratefully acknowledged.

References

1. Tsien, R. Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry* **19,** 2396–2404.
2. Linse, S., Brodin, P., Drakenberg, T., Thulin, E., Sellers, P., Elmdén, K., et al. (1987) Structure-function relationships in EF-hand Ca^{2+}-binding proteins. Protein engineering and biophysical studies of calbindin D_{9k}. *Biochemistry* **26,** 6723–6735.
3. Haugland, R. (1996) Handbook of fluorescent probes and research chemicals. Molecular Probes, Inc., Eugene, Oregon.
4. Rand, M. D., Lindblom, A., Carlson, J, Villoutreix, B. O., and Stenflo, J. (1997) Calcium binding to tandem repeats of EGF-like modules. Expression and characterization of the EGF-like modules of human Notch-1 implicated in receptor-ligand interactions. *Protein Sci.* **6,** 2059–2071.
5. Linse, S., Sellers, P., and Thulin, E. (1993) Disulfide bonds in homo and heterodimers of EF-hand subdomains of calbindin D_{9k}: stability, calcium binding and NMR studies. *Protein Sci.* **2,** 985–1000.
6. Bylsma, N., Drakenberg, T., Andersson, I., Leadley, P. F., and Forsén, S. (1992) Prokaryotic calcium-binding protein of the calmodulin superfamily. Calcium binding to *Saccharopolyspora erythraea* 20 kDa protein. *FEBS Lett.* **299,** 44–47.
7. Linse, S., Johansson, C., Brodin, P., Grundström, T., Drakenberg, T., and Forsén, S. (1991). Electrostatic contribution to the binding of Ca^{2+} in calbindin D_{9k}. *Biochemistry* **30,** 154–162.
8. Forsén, S. and Linse, S. (1995) Cooperativity: over the hill. *Tr. Biochem. Sci.* **20,** 495–497.