

# Measurement of $\text{Ca}^{2+}$ -Binding Constants of Proteins and Presentation of the CaLigator Software

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**The complexity of  $\text{Ca}^{2+}$  cell signaling is dependent on a plethora of  $\text{Ca}^{2+}$ -binding proteins that respond to signals in different ranges of  $\text{Ca}^{2+}$  concentrations. Since the function of these proteins is directly coupled to their  $\text{Ca}^{2+}$ -binding properties, there is a need for accurately determined equilibrium  $\text{Ca}^{2+}$ -binding constants. In this work we outline the experimental techniques available to determine  $\text{Ca}^{2+}$ -binding constants in proteins, derive the models used to describe the binding, and present CaLigator, software for least-square fitting directly to the measured quantity. The use of the software is illustrated for  $\text{Ca}^{2+}$ -binding data obtained for two deamidated forms of calbindin  $\text{D}_{9k}$ , either an isopartate-56 ( $\beta$  form) or a normal Asp-56 ( $\alpha$  form). Here, the  $\text{Ca}^{2+}$ -binding properties of the two isoforms have been studied using the chelator method. The  $\alpha$  form shows similar  $\text{Ca}^{2+}$ -binding properties to the wild type while the  $\beta$  form has lost both cooperativity and affinity.** © 2002 Elsevier Science (USA)

**Key Words:**  $\text{Ca}^{2+}$  binding; spectroscopy; software; computer fitting.

$\text{Ca}^{2+}$  is one of the most important and versatile intracellular messengers (1–3). It transfers information regarding nearly all aspects in the life cycle of the cell from maturation to apoptosis and regulates processes such as muscle contraction, gene expression, neuron excitation, and enzyme activity (4). As a basis for this, the cytosolic concentration of  $\text{Ca}^{2+}$  is precisely controlled, both spatially and in time, through a number of mechanisms. These involve intracellular and extracellular deposits, a number of different channels, and a set of pumps devoted to removing  $\text{Ca}^{2+}$  from the cytosol (5).

The  $\text{Ca}^{2+}$  ion is a suitable signaling substance since its binding by biological macromolecules can be modulated over a large range. The complexity of cell  $\text{Ca}^{2+}$  signaling hence depends on the presence of a multitude

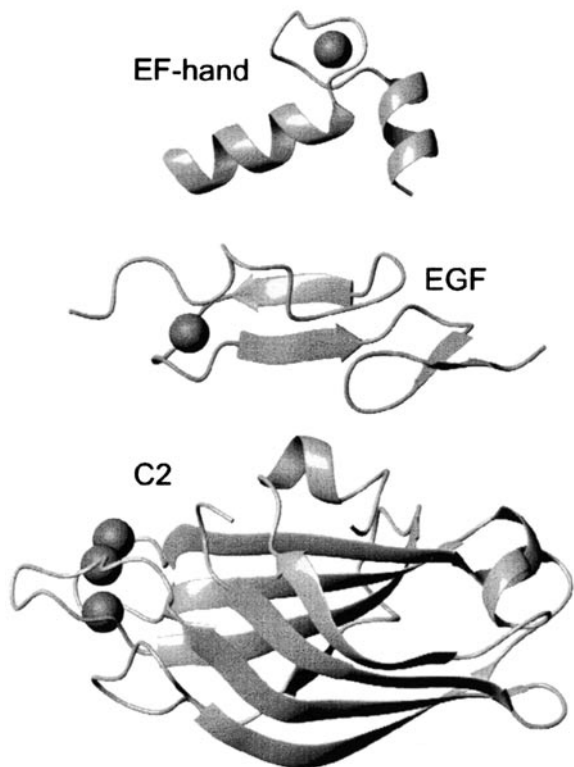
of proteins responding to different  $\text{Ca}^{2+}$  concentrations ranging from nanomolar to millimolar. Cooperative binding of  $\text{Ca}^{2+}$  by many proteins contributes to the functionality since it allows  $\text{Ca}^{2+}$  signaling to occur in a narrow concentration range (6). The  $\text{Ca}^{2+}$  binding proteins may: (i) act directly on the signal, (ii) work as  $\text{Ca}^{2+}$  sensors that interact with specific enzymes and regulate their activities, (iii) function as  $\text{Ca}^{2+}$  buffers, or (iv) transfer messages over membranes (7, 8). Extracellular  $\text{Ca}^{2+}$  is involved in bone formation, blood clotting, and cell adhesion as well as signaling (9).  $\text{Ca}^{2+}$  binding to extracellular proteins is a means of increasing their thermal stability and resistance to proteases.

Many  $\text{Ca}^{2+}$ -binding proteins belong to one of three major structural groups containing EF-hand motifs, C2 domains, or EGF<sup>2</sup>-like modules (Fig. 1). The EF-hand is a helix–loop–helix motif with one  $\text{Ca}^{2+}$  ion binding to the loop region. EF-hands are often found in pairs within proteins with up to eight EF-hands, acting as  $\text{Ca}^{2+}$  sensors, buffers, or transporters. The C2 domain is an eight-stranded  $\beta$  sandwich  $\text{Ca}^{2+}$ -binding protein module and is involved in signal transduction and membrane trafficking. C2 domains can bind to other proteins and sometimes in a  $\text{Ca}^{2+}$ -dependent manner. The EGF module contains two anti parallel  $\beta$ -sheets and three interconnecting disulfide bridges. EGF modules are found in extracellular proteins with diverse functions such as cell development, complement, and blood clotting. For extensive reviews of structures of  $\text{Ca}^{2+}$ -binding proteins we refer to some recent presentations (10, 11).

To understand the cell signaling pathway and the effect of  $\text{Ca}^{2+}$  on cellular processes it is vital to know how the proteins involved are affected by different concentrations of  $\text{Ca}^{2+}$ . Therefore, accurate values of equilibrium  $\text{Ca}^{2+}$ -binding constants are required. Apart from structural determinants, the affinity for  $\text{Ca}^{2+}$  is affected by solvent conditions such as temperature, pH, buffer strength, buffer composition, and protein concentration (12, 13). This makes it important to

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<sup>2</sup> Abbreviation used: EGF, epidermal growth factor.



**FIG. 1.** Common  $\text{Ca}^{2+}$ -binding motifs; an EF-hand, an EGF-like module, and a C2 domain. The figure was produced from the crystallographic files for calmodulin (1CLL (23)), Factor X (1CCF (24)), and phospholipase  $\text{C}\beta$  (1A25, (25)) using the program Molmol (26).

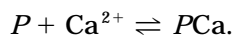
consider the exact conditions under which an experiment is conducted.

For assessment of  $\text{Ca}^{2+}$ -binding constants, it is common to start with the  $\text{Ca}^{2+}$ -free form of the protein, titrate in  $\text{Ca}^{2+}$  stepwise, and record an experimental parameter reporting on free or bound  $\text{Ca}^{2+}$ . The voltage recorded using  $\text{Ca}^{2+}$  selective electrodes reports on free  $\text{Ca}^{2+}$ , while spectroscopic techniques are convenient when the  $\text{Ca}^{2+}$ -bound and -free forms of the protein display a difference in, for example, the UV absorbance, CD, fluorescence, or NMR spectrum.

In the present work we outline different experimental techniques used to measure high- and low-affinity  $\text{Ca}^{2+}$ -binding constants. We also derive the equations used in computer fitting and introduce the software CaLigator. The use of the software is illustrated for  $\text{Ca}^{2+}$ -binding data obtained for two deamidated forms of calbindin  $\text{D}_{9k}$ .

#### Theoretical Background—Definition of Equilibrium Parameters

In the simplest case,  $\text{Ca}^{2+}$  binds to a protein with a single binding site:



We can define an equilibrium constant as

$$K = \frac{a(\text{PCa})}{a(P)a(\text{Ca}^{2+})},$$

where  $a$  is the activity. Since the activity in a protein solution is unknown, we must make the approximation that the solution can be regarded as ideal. Thermodynamic equilibrium constants are always related to a standard state and are dimensionless numbers. In biochemistry, on the other hand, binding constants are mostly calculated in terms of concentration. The affinity constant  $K_a$  is then defined as

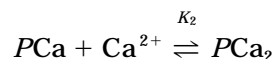
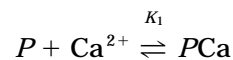
$$K_a = \frac{[\text{PCa}]}{[P][\text{Ca}^{2+}]} = \frac{1}{K_d}.$$

The dissociation constant,  $K_d$ , the inverse of  $K_a$ , is most often used. Now we can determine the standard molar reaction Gibbs free energy of binding as

$$\Delta G^\circ = -RT \ln K_a = RT \ln K_d.$$

Here, the equilibrium constant must be a dimensionless number so all concentrations in the expression for  $K_a$  must be divided by a standard state (usually 1 mol/L). The absolute value of  $\Delta G^\circ$  will depend on the definition of this standard state.

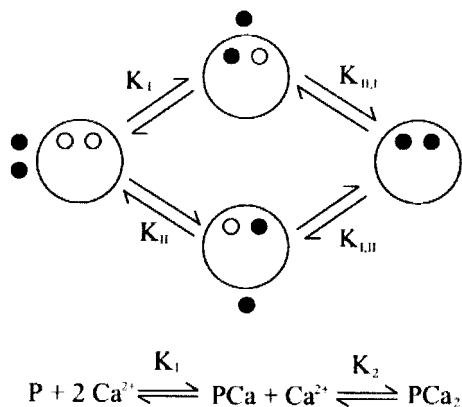
In cases with more than one binding site there will be a more complicated situation. With two binding sites, four microscopic binding constants must be used to fully describe the binding of  $\text{Ca}^{2+}$  to the protein (in reality only three of them are independent). However, with the methods and models outlined in this article only macroscopic binding constants can be determined since the response signals are only dependent on the stoichiometric number of bound  $\text{Ca}^{2+}$  per protein. The macroscopic binding constants are for the case of two binding sites are



$$K_1 = \frac{[\text{PCa}]}{[P][\text{Ca}^{2+}]} = K_{\text{I}} + K_{\text{II}}$$

$$K_2 = \frac{[\text{PCa}_2]}{[\text{PCa}][\text{Ca}^{2+}]} = \frac{K_{\text{I}}K_{\text{II,I}}}{K_{\text{I}} + K_{\text{II}}} = \frac{K_{\text{II}}K_{\text{II,II}}}{K_{\text{I}} + K_{\text{II}}},$$

where  $K_{\text{I}}$ ,  $K_{\text{II,I}}$ ,  $K_{\text{II,II}}$ , and  $K_{\text{II}}$  are microscopic binding constants (Fig. 2). In most cases, the binding of sev-



**FIG. 2.** Definition of microscopic and macroscopic  $Ca^{2+}$ -binding constants in a two-site system.

eral  $Ca^{2+}$  ions to multiple sites in a protein is not an independent event. The affinity for each site will change if other binding sites are already occupied. If the binding of one  $Ca^{2+}$  ion to a site affects the affinity for a second site, there is a cooperative binding of  $Ca^{2+}$ . The cooperativity is positive if the binding to one site increases the affinity for the second and negative if the affinity for the binding to the second site is decreased. In the case of two binding sites it is easy to see that positive cooperativity is achieved if  $K_{I,II} > K_I$  (implies that  $K_{II,I} > K_{II}$ ). The cooperativity is commonly expressed in terms of a free energy difference,  $\Delta\Delta G$ :

$$\Delta\Delta G = \Delta G_{I,II} - \Delta G_I = \Delta G_{II,I} - \Delta G_{II} = -RT \ln(K_{I,II}/K_I).$$

Expressed in macroscopic binding constants,

$$-\Delta\Delta G = RT \ln(4K_2/K_1) + RT \ln\{(\eta + 1)^2/4\eta\},$$

where  $\eta = K_{II}/K_I$ .

Defined in this way, the measure of cooperativity is still dependent on the microscopic binding constants. A lower limit to  $-\Delta\Delta G$  can be derived from the macroscopic binding constants, which is the true value of  $-\Delta\Delta G$  if  $\eta = 1$ :

$$-\Delta\Delta G_{\eta=1} = RT \ln(4K_2/K_1).$$

A more common definition of cooperativity is the Hill coefficient but it has no real physical basis (14). When more than two binding sites are present, the definition of cooperativity gets more complicated, and there may be cooperativity in some binding steps but not in others.

## MATERIALS AND METHODS

### Experimental Procedure

The choice of experimental method depends on a number of factors, such as the expected affinity and the

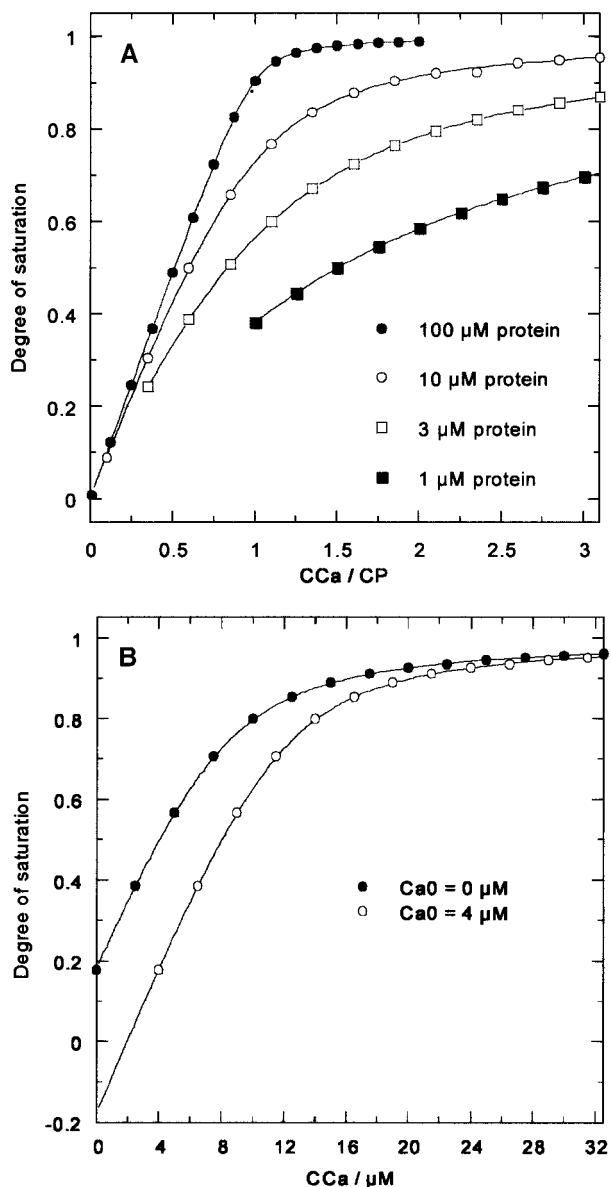
spectroscopic properties of the protein, as well as the purpose of the study. A first step may be to compare different kinds of spectra for the  $Ca^{2+}$ -free and -bound forms of the protein. If only small changes are seen, a  $Ca^{2+}$ -selective electrode may be preferred over spectroscopic titrations. In both cases, the best accuracy in the obtained binding constants 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 experimental points. However, the practical difficulty of making buffers with less than 0.5–1  $\mu M$  free  $Ca^{2+}$  limits the useful range of protein concentration to above 5–10  $\mu M$  (Fig. 3A). This means that  $Ca^{2+}$ -binding constants above ca.  $10^6 M^{-1}$  ( $K_D < 1 \mu M$ ) need to be studied using indirect measurements, for example, competition with a chelator with known affinity. One may then use a chelator whose absorbance or fluorescence is  $Ca^{2+}$  dependent. A mixture of roughly equal amounts of chelator and protein is titrated with  $Ca^{2+}$  while binding to the chelator is monitored spectroscopically. This method gives very high precision in the deduced binding constants, of special advantage when a set of proteins (e.g., mutants) are compared. The accuracy is, however, never better than the accuracy in the  $Ca^{2+}$  affinity for the chelator.  $Ca^{2+}$ -binding constants may also be studied using dialysis-based experiments (15).

A typical experimental procedure consists of the following steps.

1. Prepare the titrand. This is the starting solution of  $Ca^{2+}$ -free protein, or  $Ca^{2+}$ -free protein plus a chelator. If a  $Ca^{2+}$  electrode is used, it should be inserted in the titrand during the entire titration.
2. Spectroscopic or electrode measurement.
3. Add a  $Ca^{2+}$  aliquot. Mix using a pipette, by cuvette inversion or by automatic stirring.
4. Spectroscopic or electrode measurement.
5. Repeat steps 3 and 4 until no further change is observed in the measured parameter and at least four more times to obtain a reliable baseline.

The protein concentration in the titrand may be determined from an absorbance spectrum if the extinction coefficient is known and if the solution is nonabsorbent and free from other absorbing molecules. A more accurate way may be to perform amino acid analysis after acid hydrolysis. The initial  $Ca^{2+}$  concentration may be determined using atomic absorption or by using chromophoric  $Ca^{2+}$  chelators. The precise knowledge of the protein and initial  $Ca^{2+}$  concentration may reduce the errors in the  $Ca^{2+}$ -binding constants, depending on their value, and how critical this is may be investigated using the simulation or fitting option of the CaLigator software (Fig. 3).

Before an experiment it is advisory to keep the measuring cell in EDTA (for example 5 mM EDTA at pH 8)



**FIG. 3.** Choice of experimental conditions using the simulation option in CaLigator. We expect the binding constant to be  $10^6 \text{ M}^{-1}$  ( $\lg K = 6$ ,  $K_D = 1 \text{ } \mu\text{M}$ ), and the experimental technique will give a reasonable signal-to-noise ratio at protein concentrations between 1 and 100  $\mu\text{M}$ . A. To decide which protein concentration to use, we simulate binding curves for 1, 3, 10, and 100  $\mu\text{M}$  protein. Since we cannot expect to produce a starting solution with much less than 1  $\mu\text{M}$   $\text{Ca}^{2+}$ , we only plot data above 1  $\mu\text{M}$  total  $\text{Ca}^{2+}$ . The curve at 100  $\mu\text{M}$  protein shows that such a high protein concentration may not be useful since all the experimental points will lie close to a straight line up to 1 equivalent  $\text{Ca}^{2+}$  and then level off abruptly. The deviation from the straight line will be larger the lower the protein concentration. However, lowering the protein concentration too much means that only a portion of the curve can be covered by the experimental points. A protein concentration of somewhere around 20  $\mu\text{M}$  may be chosen as a compromise that will give the best confidence in the obtained binding constants. B. The effect of errors in the initial  $\text{Ca}^{2+}$  concentration is evaluated. Data are simulated for 10  $\mu\text{M}$  protein,  $\lg K = 6.0$  ( $K = 1.0 \times 10^6 \text{ M}^{-1}$ ), and an initial  $\text{Ca}^{2+}$  concentration ( $\text{Ca}_0$ ) of 2  $\mu\text{M}$ . These data are then fitted using  $\text{Ca}_0 = 0 \text{ mM}$  (●) or 4  $\text{mM}$  (○), yielding  $\lg K = 5.91$  ( $K = 8.2 \times 10^5 \text{ M}^{-1}$ ) and  $\lg K = 6.07$  ( $K = 1.2 \times 10^6 \text{ M}^{-1}$ ), respectively.

for 5 min and then rinse several times with doubly distilled or Millipore water and finally with ethanol and dry the cell using nitrogen gas. If this procedure is not enough to decalcify the surface of the cell, one may perform a quick wash using 1:3:4 HF:HNO<sub>3</sub>:H<sub>2</sub>O followed by extensive rinsing to remove the acid.

A  $\text{Ca}^{2+}$  titration experiment ideally starts with a completely  $\text{Ca}^{2+}$ -free protein solution. In practice this is very difficult to achieve, especially with high-affinity  $\text{Ca}^{2+}$ -binding proteins.  $\text{Ca}^{2+}$  may be removed from the protein by passing the sample through a Chelex column or by mixing it with an excess of a chelating agent like EDTA or EGTA, which is then removed by gel filtration. In both cases pH is an important parameter because the  $\text{Ca}^{2+}$  affinity for the Chelex or chelators increases steeply with increasing pH, while many proteins have an affinity that is less pH sensitive between 6.5 and 9, especially if the protein contains no histidines. A pH around 8 may be a good compromise between efficient  $\text{Ca}^{2+}$  removal and protein stability. The decalcified protein must be eluted into plastic vials or tubes or into glassware that has been acid washed and then extensively rinsed to remove the acid.

Also, the preparation of the buffer needs special precautions to obtain a low residual  $\text{Ca}^{2+}$  concentration. It is often best to avoid glass containers completely and to store buffers with a dialysis bag containing Chelex-100 resin (Bio-Rad) inside.

## RESULTS AND DISCUSSION

### *Extraction of $\text{Ca}^{2+}$ -Binding Constants from Experimental Data*

The raw data from a  $\text{Ca}^{2+}$  titration experiment is a series of recordings (absorbance, chemical shift, electrode voltage, etc.) taken at different total  $\text{Ca}^{2+}$  concentrations. It is often convenient to start the data analysis by plotting these data points in a simple  $x$ - $y$  plot. The location of the points on this plot is governed by the  $\text{Ca}^{2+}$ -binding constants in the studied system and a few other factors depending on the type of experiment performed: protein concentration, the recordings that would arise in completely  $\text{Ca}^{2+}$ -free and  $\text{Ca}^{2+}$ -saturated solutions, or electrode calibration curve and offset. Based on a relevant binding model, one may derive an equation that reproduces the data points. The binding constants and other factors are introduced in this equation as adjustable parameters. By comparing the curves obtained with different combinations of parameter values, one may choose the one that best agrees with the obtained data to obtain estimates of the binding constants and other factors. This is most efficiently done using a computer program, which enables the testing of many different combinations of parameter values and iteration of the parameters in a strategic way to rapidly converge to the best solution, so-called computer fitting of data.

Traditionally, binding constants were instead obtained by transforming the raw data to a form reproduced by a straight line, because the transformation and fitting could be done by hand. Examples of such transformations were Scatchard and Hill plots. However, the transformation required that some parameters were already known and the end data points were used to transform all other points. This introduced systematic errors. In addition, the transformation procedure invoked uneven weighting of the data points and a few data points tended to guide the fit although these points were not measured by higher precision than the others. Still, over 15 years after common access to computers, the Scatchard and Hill plots are often seen in the literature. One reason is that this is the way binding analysis is presented in many textbooks. Another reason may be that not all scientists have the habit of typing the relevant equations into curve fitting software or of writing his/her own software.

The CaLigator software has been developed as a user-friendly environment where equilibrium binding constants can be determined from  $\text{Ca}^{2+}$  titration experiments through fitting to the measured quantity. The software handles a number of different experimental setups involving  $\text{Ca}^{2+}$ -selective electrodes, spectroscopy, and/or chromophoric  $\text{Ca}^{2+}$  chelators. We have included in the program a simulation option so that the user can easily learn how the binding constants and other parameters affect the obtained curve. The simulation option is also useful both when planning the experimental setup and when finding starting parameters for the fitting routine.

### Curve Fitting

CaLigator uses a Levenberg–Marquardt nonlinear fitting routine (16) to find the best matched curve. As the measure of the goodness-of-fit the quantity  $\chi^2$  is used,

$$\chi^2(\mathbf{a})^2 = \sum_{i=1}^N \left[ \frac{y_i - y(x_i, \mathbf{a})}{\sigma_i} \right]^2,$$

where  $y$  is the experimental response signal,  $x$  is the  $\text{Ca}^{2+}$  concentration,  $y(x, \mathbf{a})$  is the model function,  $\mathbf{a}$  is the parameter set, and  $\sigma$  is the standard deviation. The  $\chi^2$  distribution is a sum of  $N$  squared normally distributed quantities, and the measured property is assumed to have normally distributed errors (an assumption that is not always true). The problem of finding the best fit is then reduced to the mathematical problem of minimizing  $\chi^2$ . In the equation for  $\chi^2$ , individual standard deviations,  $\sigma_i$ , must be specified. The CaLigator software enables an estimated relative standard deviation

in percent ( $e$ ) in the measured quantity and calculates the individual standard deviations as

$$\sigma_i = y_i * e / 100.$$

These standard deviations are needed to estimate confidence limits of the parameters in the model. If no error is specified, or if it is unknown, the goodness-of-fit is instead validated by the Error-Square-Sum (E.S.S.):

$$\text{E.S.S.} = \sum_{i=1}^N [y_i - y(x_i, \mathbf{a})]^2.$$

This will not give exactly the same result as using  $\chi^2$ , since E.S.S. can be seen as  $\chi^2$  with the individual  $\sigma_i$  set to 1 and will hence not weigh the points in the same manner. The differences are, however, small if the errors are of similar magnitude throughout the experiment. The software also allows for constant standard deviations to be used. The Levenberg–Marquardt method elegantly uses a combination of the deepest descent and the inverse Hessian method. When far away from the minimum, it uses the deepest descent, and in close proximity to the minimum, the inverse Hessian method is used. The partial derivatives of  $y(x_i, \mathbf{a})$  are calculated numerically, and the binding equations are solved iteratively using the Newton–Raphson method to find the free  $\text{Ca}^{2+}$  concentration. If the errors in the parameters do not extend outside a region where the model could be replaced with a linearized model the errors can be calculated as

$$da_i = \sqrt{\Delta\chi^2 C_{ii}^2}$$

where  $\Delta\chi^2$  ( $\Delta\chi^2 = 1$  for one standard deviation) is the difference between some arbitrary value of  $\chi^2$  and the value at the found minimum,  $\chi_{\min}^2$ , and  $C_{ii}$  are the elements of the covariance matrix. This means that the sample size must be sufficiently large to get a valid estimation of the errors. It should be noted that the equation will only give a confidence interval for a single parameter, and this is different from the confidence interval for a number of parameters jointly (for example, the confidence limit for  $K_1$  and  $K_2$  jointly in a two-binding-site model). Systematic errors are not included in the error estimate. Another option to estimate the confidence limits is to use the jackknife method to estimate the errors. Here, the dataset is reduced with about 10% and the data are refitted. This procedure is repeated 50 times and from the resulting parameter sets the standard deviations can be calculated. This method has the advantage that it does not require any extra information to calculate confidence limits. However, the method will perform better for large datasets. A good way to estimate confidence in-

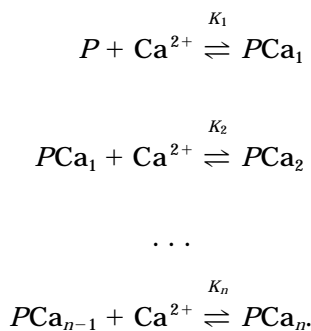
tervals is to make multiple experiments and compare the fitted parameter values.

One problem of fitting data to complex nonlinear equations is that the fitting routine can converge to a local minimum. The best way to force the solution into the global minimum is to give the fitting routine good initial start values of the parameters. Since there is no mathematical way to establish that the global minimum has been reached, critical assessments of the fitted parameters and the graphical output are always necessary.

### Binding Models

The models supplied with CaLigator give the opportunity to calculate binding constants for models with up to six binding sites using a  $\text{Ca}^{2+}$ -selective electrode or the chelator method. There are specific models for one, two, four, and six  $\text{Ca}^{2+}$  sites. To calculate binding constants with three or five binding sites, a model with four or six binding sites, respectively, can be used with the fourth or sixth binding constant locked to a very small value. Also supplied are models with one and two binding sites that can be used if a direct signal (fluorescence, NMR chemical shift, etc.) is collected from the binding events. Residual concentration of a chelator (e.g., EGTA or EDTA) used to remove  $\text{Ca}^{2+}$  from the protein stock can be treated in a model with two binding sites and two chelators.

The general model is used to describe the binding events:



The total  $\text{Ca}^{2+}$  concentration at each titration point is the sum of free  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  bound to the protein:

$$[\text{Ca}^{2+}]_{\text{tot}} = [\text{Ca}^{2+}]_{\text{free}} + [\text{PCa}]_1 + 2[\text{PCa}]_2 + \dots + n[\text{PCa}]_n. \quad [1]$$

The total protein concentration is distributed over species with different numbers of bound  $\text{Ca}^{2+}$  ions:

$$[P]_{\text{tot}} = [P]_0 + [\text{PCa}]_1 + [\text{PCa}]_2 + \dots + [\text{PCa}]_n. \quad [2]$$

The macroscopic binding constants are generally described by

$$\begin{aligned} K_i &= \frac{[\text{PCa}_i]}{[\text{PCa}_{i-1}][\text{Ca}]_{\text{free}}} \\ &\Rightarrow [\text{PCa}_i] = K_i[\text{PCa}_{i-1}][\text{Ca}]_{\text{free}} \\ &\Rightarrow [\text{PCa}_i] = [P]_0 \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}}. \end{aligned} \quad [3]$$

If we use [3] in [1] we obtain

$$[\text{Ca}^{2+}]_{\text{tot}} = [\text{Ca}^{2+}]_{\text{free}} + [P]_0 \sum_{i=1}^N i \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}} \quad [4]$$

and using [3] in [2] we obtain

$$\begin{aligned} [P]_{\text{tot}} &= [P]_0 + [P]_0 \sum_{i=1}^N \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}} \\ &\Rightarrow [P]_0 = \frac{[P]_{\text{tot}}}{1 + \sum_{i=1}^N \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}}}. \end{aligned} \quad [5]$$

Then using [5] in [4] we obtain

$$[\text{Ca}^{2+}]_{\text{tot}} = [\text{Ca}^{2+}]_{\text{free}} + \frac{[P]_{\text{tot}} \sum_{i=1}^N i \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}}}{1 + \sum_{i=1}^N \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}}}. \quad [6]$$

In the simplest case of only one binding constant, we have

$$[\text{Ca}^{2+}]_{\text{tot}} = [\text{Ca}^{2+}]_{\text{free}} + \frac{[P]_{\text{tot}} K [\text{Ca}]_{\text{free}}}{1 + K [\text{Ca}]_{\text{free}}}. \quad [7]$$

From this equation, we can obtain an analytical solution of the free  $\text{Ca}^{2+}$  concentration as

$$\begin{aligned} [\text{Ca}^{2+}]_{\text{free}} &= -\frac{1}{2} ([P]_{\text{tot}} + K^{-1} - [\text{Ca}^{2+}]_{\text{tot}}) \\ &\quad + \sqrt{\frac{1}{4} ([P]_{\text{tot}} + K^{-1} - [\text{Ca}^{2+}]_{\text{tot}})^2 + [\text{Ca}^{2+}]_{\text{tot}} K^{-1}}. \end{aligned} \quad [8]$$

In most other cases, the free  $\text{Ca}^{2+}$  concentration is obtained using numerical methods, for example, the Newton–Raphson method.

### Equations for a $\text{Ca}^{2+}$ Selective Electrode

If a  $\text{Ca}^{2+}$ -selective electrode is used the signal,  $V$ , is linearly dependent on the logarithm of the free  $\text{Ca}^{2+}$  concentration,

$$V_{\text{calc}} = k * \log[\text{Ca}^{2+}]_{\text{free}} + m, \quad [9]$$

where  $k$  is a proportionality coefficient, which is determined through calibration of the electrode, and  $m$  is an offset that can vary between experiments. Before Eq. [9] can be used for fitting to electrode data,  $[\text{Ca}^{2+}]_{\text{free}}$  must be replaced by the right-hand side of Eq. [8] if the protein has only one binding site or by a numerical solution if the binding model is more complex.

### Equations for Spectroscopic Measurements

If the measured signal is directly affected by the binding of  $\text{Ca}^{2+}$  to a protein the signal can be described by

$$S_{\text{calc}} = (f_0 * S_0 + f_1 * S_1 + \dots + f_n S_n) \frac{C_{p,i}}{C_{p,0}}, \quad [10]$$

where  $f_i$  is the fraction protein molecules that have  $i$   $\text{Ca}^{2+}$  ions bound,

$$f_i = [PCa_i] / C_{p,i}, \quad [11]$$

and  $S_i$  is the signal generated by this fraction. The factor  $C_{p,i} / C_{p,0}$  in Eq. [10] corrects for signal loss due to dilution of the protein upon  $\text{Ca}^{2+}$  addition, which occurs in all types of spectroscopic titrations where a signal intensity is measured. However, if, for example, NMR chemical shift or fluorescence polarization is measured, this factor should be omitted:

$$S_{\text{calc}} = (f_0 * S_0 + f_1 * S_1 + \dots + f_n S_n). \quad [12]$$

### Equations for Spectroscopic Measurements Using Chromophoric Chelators

If a chelator is competing for  $\text{Ca}^{2+}$ , an extra term describing chelator-bound  $\text{Ca}^{2+}$  is included in the equation for total  $\text{Ca}^{2+}$  concentration. Equation [6] transforms into

$$[\text{Ca}^{2+}]_{\text{tot}} = [\text{Ca}^{2+}]_{\text{free}} + \frac{[\text{Ca}]_{\text{free}}[\text{chelator}]_{\text{tot}}}{K_d + [\text{Ca}]_{\text{free}}} + \frac{[P]_{\text{tot}} \sum_{i=1}^N i \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}}}{1 + \sum_{i=1}^N \prod_{j=1}^i K_j [\text{Ca}]_{\text{free}}}, \quad [13]$$

where  $[\text{chelator}]_{\text{tot}}$  is the total chelator concentration and  $K_d$  is the dissociation constant for the chelator- $\text{Ca}^{2+}$  complex.

In the chelator method, the absorbance or fluorescence signal at each recording is calculated as

$$a_{\text{calc}} = \left( a_{\text{free}} + (a_{\text{bound}} - a_{\text{free}}) \frac{[\text{Ca}^{2+}]_{\text{free}}}{K_d + [\text{Ca}^{2+}]_{\text{free}}} \right) \frac{C_{q,i}}{C_{q,0}}, \quad [14]$$

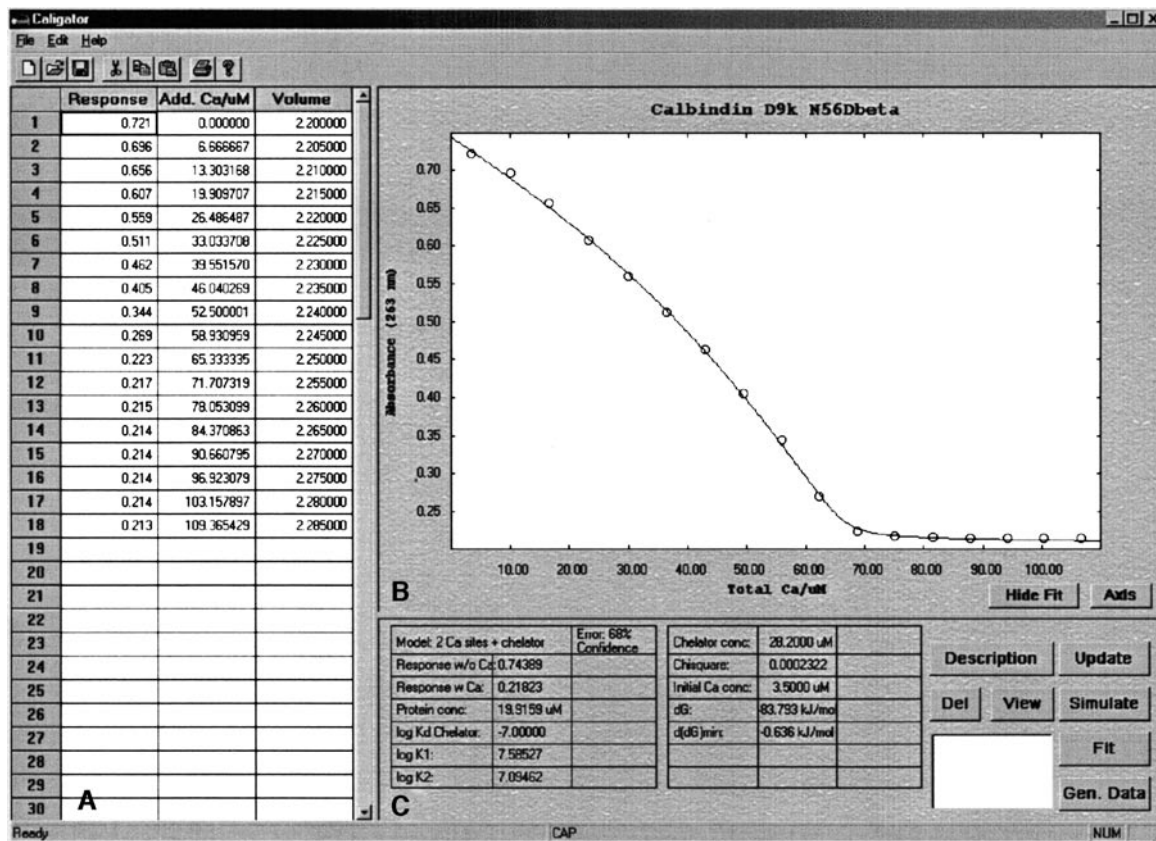
where  $C_{q,0}$  and  $C_{q,i}$  are the total protein concentrations at the start and at titration point  $i$ , respectively. During the fitting procedure,  $[\text{Ca}]_{\text{free}}$  in Eq. [14] is replaced by a numerical solution of Eq. [13].

### Using the Caligator Software

*Data input.* There are several ways to input data to the software. Data can be directly entered into a data sheet where basic operations can be performed. If a more complex data processing is needed this can be done in appropriate external software and the data can then be pasted into CaLigator through the common clipboard. The inserted  $\text{Ca}^{2+}$  concentrations should be compensated for dilution because of increased volume. To take dilution effects into account the software also needs the total volumes at each titration point. To simplify the process of calculating all concentrations at each titration point, the user has the option of generating this data inside the software. By specifying a start volume and the number of additions of a certain volume and concentration the user can sequentially add data to the sheet and the dilution-compensated concentrations are automatically calculated.

*Simulation.* The simulation tool has three purposes. First, good starting values for the fitting routine can be found by setting parameters giving a close match to the titration curve. Second, the tool can be used to study the importance of different parameters and to see how they influence the binding curve. Also, this tool can be used as an experimental planner. If approximate values of the model are known, the experimental settings that give the best conditions for finding the parameters can be found. The best way to design the experiment is described under Experimental Procedures. In the simulation dialog the appropriate model must be selected first. Then the values of the parameters are set. It is also possible to select whether the experimental response-signal is affected by dilution (intensity properties are always affected by dilution). When a reasonable match between the titration curve and the simulated curve is found the parameters can be copied to the fit dialog to find the parameters of the model.

*Fitting.* The fit dialog has the same buildup as the simulation dialog. First the model is selected and then start values for the fitting routine are set. If some of the parameters are known with accuracy these parameters will not have to be fitted and this will increase the accuracy of the fit. Such parameters can be held con-



**FIG. 4.**  $\text{Ca}^{2+}$  titration data of N56D $\beta$  in 28.2  $\mu\text{M}$  Br<sub>2</sub>-BAPTA as viewed in the Caligator software. Data are inserted in the data view (A) and displayed in the graphic view (B). The result of the computer fitting is seen in output view (C).

stant in the fit. As in the simulation dialog, it is possible to specify whether the response signal is affected by dilution. If the standard deviation is wanted it is possible to specify the standard deviation/relative standard deviation in the measured quantity. Estimated confidence limits (at one standard deviation, that is at 68.3% certainty) of all parameters are then also calculated. The result is presented in the output view and the fitted curve is drawn in the graph view. Since there is a risk of finding a local minimum, the graph view must be consulted to assure a good match between experimental points and fitted curve and the user should check that the parameters are reasonable. If the protein concentration is more than 20% higher than the starting value the software will give a notification of this since this is an indication that a false minimum has been found. If a good fit is not found the user is advised to change some start parameters or go to the simulation tool.

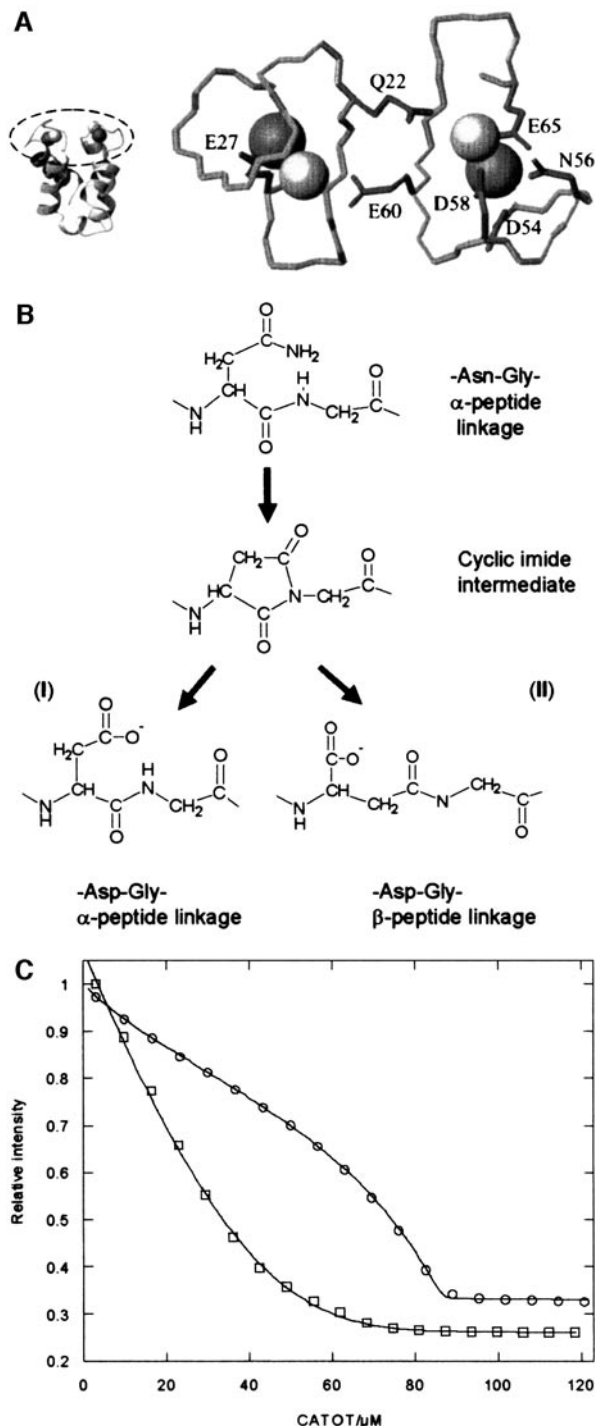
**Output.** The output view of the software gives the values of the fitted parameters and also the 68.3% confidence limit if a standard deviation was specified or the jackknife method was used. The result and the graphic plot including simulated curves can be plotted together with an experimental description. For more

advanced graphic representation of the curves and the data these can be exported in ASCII format and imported into other software packages. It is also possible to export saturation curves, where fraction saturated molecules is plotted against free  $\text{Ca}^{2+}$  concentration.

**Deamidation of calbindin D<sub>9k</sub>.** The use of the CaLigator software is here exemplified by deamidated forms of calbindin D<sub>9k</sub>. Deamidation is a posttranslational conversion of asparagine residues. Deamidation of glutamine residues is also possible but less probable. Deamidation of asparagines occurs through an intramolecular cyclization that leads to the formation of either an isoaspartate or an aspartate (Fig. 5B). If an asparagine is followed by a glycine in the amino acid sequence either an  $\alpha$ - or a  $\beta$ -linkage (isoaspartyl linkage) can be formed (17, 18). Formation of an isoaspartyl linkage leads to the insertion of an extra  $\text{CH}_2$  in the backbone. Together with the charge difference, the lengthening of the backbone can seriously perturb the structure and function of a protein.

Calbindin D<sub>9k</sub> belongs to the EF-hand superfamily and contains two  $\text{Ca}^{2+}$ -binding sites. It is prone to deamidation at residue Asn 56, which is followed by a Gly in the amino acid sequence (19). Asn 56 is part of the  $\text{Ca}^{2+}$ -binding loop of the second EF-hand in cal-





**FIG. 5.** Deamidation. **A.** 3D structure of Calbindin  $D_{9k}$  showing the location of Asn56, which is prone to deamidation. The figure was produced from the crystallographic file 4icb (20) using the program Molmol (26). **B.** The Asn-Gly linkage can be transformed to either a Asp-Gly  $\alpha$ -peptide linkage (I) or a Asp-Gly  $\beta$ -peptide linkage (II) through cyclic imide intermediate [after (19)]. **C.**  $Ca^{2+}$  titration data of N56D $\alpha$  and N56D $\beta$  in quin 2 together with computer fitting. (○) Titration of N56D $\alpha$  in 30.0  $\mu$ M quin 2. (□) Titration of N56D $\beta$  in 30.6  $\mu$ M quin 2.

bindin  $D_{9k}$  (Fig. 5A) and Asn 56 is the  $X$  ligand in the coordination nomenclature of EF-hands. The introduction of an aspartate at position 56 creates a sequence

with three negatively charged aspartates (Asp 54, Asp 56, Asp 58) at coordinates  $X$ ,  $Y$ , and  $Z$ . The inserted Asp will then function as a ligand to the  $Ca^{2+}$ . The effect on the macroscopic  $Ca^{2+}$ -binding constants due to the deamidation of Asn56 was studied using the chelator method (Figs. 4 and 5C).

The  $\alpha$ -deamidated form of calbindin  $D_{9k}$  was prepared by the mutation Asn 56  $\rightarrow$  Asp, expressed in *Escherichia coli* and purified as described (20). The  $\beta$  form was isolated after forced deamidation of the wild-type protein. First, the remaining undeamidated material was removed using ion exchange chromatography as described (19). The two deamidated forms were then separated using preparative isoelectric focusing on an LKB 2117 Multiphor II electrophoresis unit using a homecast gel with a pH gradient from 3.5 to 5. A total of 5.5 mg of each protein was decalcified in 3.3 mM EDTA, pH 8.0, followed by desalting on a NAP-10 column from Pharmacia Biotech (Uppsala, Sweden). The macroscopic  $Ca^{2+}$ -binding constants were determined with the chelator method using either quin 2 (Fluka, Switzerland) or 5,5'-Br<sub>2</sub>-BAPTA (Molecular Probes, Eugene, OR). The wild-type and the deamidated forms were titrated with  $Ca^{2+}$  in the presence of quin 2 (30.0 and 30.6  $\mu$ M) and the  $\beta$  form in the presence of 5,5'-Br<sub>2</sub>-BAPTA (28.1  $\mu$ M) at a protein concentration of 20–25  $\mu$ M in 2 mM Tris/HCl, pH 7.5, buffer at 25°C. The chelator concentration was determined by measuring the absorbance at 239.5 nm, using  $\epsilon_{239.5} = 4.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  for quin 2 and  $\epsilon_{239.5} = 1.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  for 5,5'-Br<sub>2</sub>-BAPTA. The concentration of the  $Ca^{2+}$  stock was determined by ICP-AES. All chemicals were of the highest purity commercially available.

The  $Ca^{2+}$  affinity of the  $\alpha$  form has not changed significantly compared to the wild type (Fig. 5C, Table 1). Binding still occurs with positive cooperativity and this can of course occur only if both sites are still active.

**TABLE 1**

Calcium-Binding Constants for Calbindin  $D_{9k}$  N56D $\alpha$  and N56D $\beta$  Together with  $\Delta G_{\text{tot}}$  for Calcium Binding and Cooperativity  $\Delta \Delta G_{\eta=1}$ :  $\Delta G_{\text{tot}} = -RT \ln(K_1 K_2)$ ,  $\Delta \Delta G_{\eta=1} = -RT(4K_2/K_1)^a$

	(M <sup>-1</sup> )		(kJ/mol)	
	$K_1$	$K_2$	$\Delta G_{\text{tot}}$	$\Delta \Delta G_{\eta=1}$
N56D $\alpha$ , quin 2	$3.0 \times 10^8$	$4.7 \times 10^8$	-97.5	-4.5
N56D $\beta$ , quin 2 <sup>b</sup>	$6.6 \times 10^7$	$0.6 \times 10^7$	-83.3	2.5
N56D $\beta$ , Br <sub>2</sub> -BAPTA	$3.9 \times 10^7$	$1.2 \times 10^7$	-83.8	-0.6

<sup>a</sup> Previously  $K_1$  and  $K_2$  have been determined to be  $1.6 \times 10^8$  and  $4.0 \times 10^8 \text{ M}^{-1}$  for the  $\alpha$  form and  $2.0 \times 10^7$  and  $3.2 \times 10^7 \text{ M}^{-1}$  for the  $\beta$  form (19). However, these measurements were done on a mixture of  $\alpha$  and  $\beta$  forms, which may explain the differences.

<sup>b</sup> Even though the exact values of  $K_1$  and  $K_2$  are uncertain, the product  $K_1 K_2$  is the same as in the Br<sub>2</sub>-BAPTA experiment as seen by the  $\Delta G_{\text{tot}}$  values.

NMR data (19) show that the global conformation and secondary structure of the  $\alpha$  form have not changed markedly compared to the wild type.

Iso-Asp 56 calbindin D<sub>9k</sub> (the  $\beta$  form) has considerably lower Ca<sup>2+</sup>-binding constants, and the data obtained with quin 2 suggest that both log  $K_1$  and log  $K_2$  may be lower than 7 (Fig. 5C). Hence, quin 2 is not a suitable chelator with its log  $K$  of 8.28 and an accurate determination of  $K_1$  and  $K_2$  is not possible. Instead, the titration was repeated with 5,5'-Br<sub>2</sub>-BAPTA that has a log  $K$  of 7.00, and it appears that both  $K_1$  and  $K_2$  are lowered considerably and are in the same range (Fig. 4). The ratio  $K_2/K_1$  is close to 0.25, which could occur for two sites with the same affinity but no cooperativity. In such a case there is a risk of misinterpretation of the data, as twice the protein concentration and a single binding site would produce identical data. To distinguish between these two cases, the protein concentration was measured using amino acid analysis after acid hydrolysis. The obtained concentration, 19.1  $\mu$ M, is within error limits in agreement with the result of the fit (19.5  $\mu$ M) using two binding sites and  $K_2 \approx 0.25 K_1$ . It can therefore be concluded that in the  $\beta$  form, the Ca<sup>2+</sup> affinity is significantly reduced for both sites and there is no apparent cooperativity.

The introduction of the isoaspartyl linkage seriously affects the Ca<sup>2+</sup>-binding properties of both sites as well as the coupling between them. The very short  $\beta$ -Asp side chain may not be able to establish a favorable O-Ca<sup>2+</sup> coordination distance. To accommodate the extra CH<sub>2</sub> group the structure of the second EF-hand is perturbed. The NMR data of the  $\beta$  form show that the largest changes occur in the polypeptide segment containing Lys 55, Asp 56, Gly 57, and Asp 58 (19) and the binding data indicate that these structural changes are propagated to the other site. The NMR chemical shifts are, however, mainly affected for residues 50–70, with small changes for residues 23 and 25 (19), suggesting that the geometry of the N-terminal site is virtually intact. Therefore, it is most likely that the affinity of the N-terminal site has decreased due to perturbed contacts with the C-terminal site, an idea that is supported by the fact that EF-hand homodimers have significantly lower Ca<sup>2+</sup> affinity than the native protein (21). These perturbations may involve the  $\beta$ -sheet between the two sites, the symmetric bridging of Gln 22 and Glu 60 that stabilize Ca<sup>2+</sup>-coordinating water molecules in opposite sites, or the hydrophobic core packing of the two EF-hands. The introduction of an extra negative charge normally yields a higher cooperativity since most of the free energy penalty of bringing the negatively charged groups closer together is paid for in the first binding step. It seems, however, that the combined effects lead to a diminished cooperativity.

*Conditions for use.* The CaLigator software is free of charge for academic users and can be downloaded at [http://www.bpc.lu.se/staff/personal/ingemar\\_andre.html](http://www.bpc.lu.se/staff/personal/ingemar_andre.html). The only requirement is that you make a reference to the software in any publication that makes use of CaLigator.

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