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Quantitative measurements of the cooperativity in an EF-hand protein with sequential calcium binding

SARA LINSE[1] AND WALTER J. CHAZIN[2]

[1] Department of Physical Chemistry 2, The Chemical Centre, University of Lund, S-221 00 Lund, Sweden
[2] Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

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Abstract

Positive cooperativity, defined as an enhancement of the ligand affinity at one site as a consequence of binding the same type of ligand at another site, is a free energy coupling between binding sites. It can be present both in systems with sites having identical ligand affinities and in systems where the binding sites have different affinities. When the sites have widely different affinities such that they are filled with ligand in a sequential manner, it is often difficult to quantify or even detect the positive cooperativity, if it occurs. This study presents verification and quantitative measurements of the free energy coupling between the two calcium binding sites in a mutant form of calbindin D_{9k}. Wild-type calbindin D_{9k} binds two calcium ions with similar affinities and positive cooperativity—the free energy coupling, $\Delta\Delta G$, is around -8 kJ·mol^{-1} (Linse S, et al., 1991, *Biochemistry 30*: 154–162). The mutant, with the substitution Asn 56 → Ala, binds calcium in a sequential manner. In the present work we have taken advantage of the variations among different metal ions in terms of their preferences for the two binding sites in calbindin D_{9k}. Combined studies of the binding of Ca^{2+}, Cd^{2+}, and La^{3+} have allowed us to conclude that in this mutant $\Delta\Delta G < -6.4$ kJ·mol^{-1}, and that Cd^{2+} and La^{3+} also bind to this protein with positive cooperativity. The results justify the use of the $(Ca^{2+})_1$ state of the Asn 56 → Ala mutant, as well as the $(Cd^{2+})_1$ state of the wild type, as models for the half-saturated states along the two pathways of cooperative Ca^{2+} binding in calbindin D_{9k}.

Keywords: Ca^{2+} binding; calbindin D_{9k}; cooperativity; free energy coupling; ^{1}H NMR

Cooperativity is one of the most fascinating functional properties of biological systems. It is highly economic for the living cell in that it narrows the concentration interval of free ligand over which the regulation is accomplished. Monod is said to have referred to cooperativity as the "second secret of life," the structure of DNA being the first (Perutz, 1989). Positive cooperativity of ligand binding occurs in a wide variety of systems with the size of the ligand ranging from small ions to large proteins.

Calcium binding proteins (CABPs) are crucial to many intracellular and extracellular processes, including regulation of transcription and cellular enzymes, muscle contraction, tooth mineralization, blood coagulation, transmission of sensory signals, as well as protection against neuronal cell death (Heizmann & Hunziker, 1990; Davie et al., 1991; Baimbridge et al., 1992; Andressen et al., 1993; Filipek et al., 1993). The ability of a CABP to regulate or be regulated by the free Ca^{2+} concentration is in most cases central to its function. Some CABPs can, like calmodulin, transmit the signal of elevated free calcium to regulation of other proteins, whereas the role of others is to buffer the free calcium in a certain concentration interval and, maybe more important, on a particular time scale (Williams, 1992). In both classes, Ca^{2+} buffers and Ca^{2+} regulatory proteins, the performance is much enhanced if the CABP is capable of binding Ca^{2+} ions with positive cooperativity.

We have chosen calbindin D_{9k} as a model system for studying the biophysics and molecular mechanism of cooperative calcium binding. Like the majority of CABPs, calbindin D_{9k} belongs to the calmodulin superfamily, which is distinguished by a highly conserved helix-loop-helix motif called the EF-hand (Kretsinger & Nockolds, 1973). Structural studies have shown that EF-hands are most often arranged in pairs, or higher order assemblies, with a short β-type interaction between the two 12-residue Ca^{2+}-binding loops. Pairs of EF-hands, either isolated or in the context of a larger globular domain, have repeatedly been found to bind Ca^{2+} ions with positive cooperativity, as for example in parvalbumin (Cave et al., 1979; Moeschler et al., 1980), troponin C (Grabarek et al., 1983; Teleman et al., 1983; Pearlstone et al., 1992), calmodulin (Crouch & Klee, 1980; Linse et al., 1990), calbindin D_{9k} (Linse et al., 1991), recoverin (Zozulya & Stryer, 1992), calerythrin (Bylsma et al., 1992), and

Reprint requests to: Sara Linse, Department of Physical Chemistry 2, The Chemical Centre, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden; e-mail: sara@freja.fkem2.lth.se.

sarcoplasmic calcium binding protein (Cox & Stein, 1981). However, gaining an understanding of the molecular mechanism underlying the observed cooperativity is an elaborate task involving detailed characterization of all ion ligation states. The binding processes in a typical two-site domain are shown in schematic form in Figure 1. Particular insights in this system would be gained if one could analyze the structure and dynamics of states with only one calcium ion bound, in comparison with data for the Ca^{2+}-free (apo) and Ca^{2+}-loaded forms. But, due to the cooperativity, these half-saturated states are populated at too low levels in any equilibrium mixture to allow direct experimental observations. One way around this problem is to use a substitution probe for calcium: for example, another metal ion that has different affinities for the two sites so that these are filled in a sequential manner. An alternative approach is to selectively reduce the Ca^{2+} affinity of one of the sites by mutation; to be effective, this mutation should have little or no effect on the affinity for the other site. The former strategy is exemplified in the extensive high-resolution NMR studies of calbindin D_{9k} with a cadmium ion occupying site II only (Akke et al., 1991, 1993, 1995; Skelton et al., 1992). Examples of the latter approach are given by E65Q (Carlström & Chazin, 1993) and N56A (the mutants examined in this study), both of which are designed to have a reduced calcium affinity at the C-terminal site (site II) such that the form with only one calcium ion bound (in the N-terminal site I) can be studied.

A critical requirement of these models of the inaccessible wild-type $(Ca^{2+})_1$ states is that the substitution (of the ion or of an amino acid) does not substantially alter the cooperativity. Because the cooperativity is defined simply as a free energy coupling between the binding sites, it is just as likely to occur in a system that binds two ions in a sequential manner. However, in the sequential binding system, there is a problem of experimental verification. For example, in the mutants that bind calcium sequentially, we can only measure the affinity of the weaker site in the presence of a Ca^{2+} ion in the stronger site, not in its absence. Likewise, we can only measure the affinity for the stronger site when the other site is empty. It is therefore not possible to establish through direct experiments whether calcium binding to one site alters the calcium affinity for the other site.

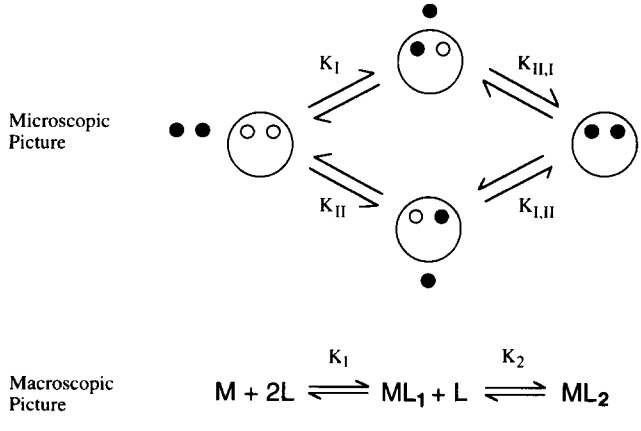

Fig. 1. Ligand binding equilibria in a system with two sites for the same type of ligand. The microscopic and macroscopic binding constants are in the respective pictures.

In the present work, we have taken advantage of the variations in binding site preferences of different metal ions to assess the free energy coupling between binding events at the two sites in the N56A mutant. The same strategy also allows us to address the question of whether cadmium ions bind to calbindin D_{9k} with positive cooperativity. In the case of wild-type calbindin D_{9k}, most lanthanides bind to site II with three to six orders of magnitude higher affinity than to site I (Hoffman et al., 1988). The same order of binding holds for cadmium ions, although the difference between the sites is only around two orders of magnitude. With the exception of lanthanum and lutetium, all lanthanide ions are paramagnetic and give rise to severe line-broadening of NMR spectra. In the present work we have therefore chosen to study the binding of cadmium and lanthanum ions to N56A. Because it is possible to produce a $(Ca^{2+})_1$ state of this mutant, our approach has been to measure binding of La^{3+} and Cd^{2+}, respectively, both in the absence and presence of Ca^{2+} in site I.

Results

The first objective was to carefully determine the Ca^{2+} binding properties of the N56A mutant, which required two different methods: (1) Ca^{2+} titrations monitored by ^1H NMR, and (2) Ca^{2+} titrations in the presence of a chromophoric chelator monitored by UV absorbance. The second objective involved the assessment of the Cd^{2+} and La^{3+} binding properties of the N56A mutant in the complete absence of calcium. To complete the analysis, the Cd^{2+} and La^{3+} binding properties of the mutant were measured with a calcium ion present in site I.

Ca^{2+} binding

Initial characterization of the calcium binding properties of N56A was made by one-dimensional ^1H NMR. Spectra were acquired for 2 mM N56A, in the absence of calcium and after stepwise additions of ca 0.10 equivalents of calcium. Sample spectra from this titration are shown in Figure 2. At additions up to 1.0 equivalent, new resonances appear and increase in intensity, whereas others decrease in intensity as a function of added calcium. This observation is indicative of a slow exchange process corresponding to Ca^{2+} binding at site I. At additions between 1.0 and slightly more than 2.0 equivalents, no new resonances are observed, but several signals move toward higher or lower shifts as a function of added calcium. A slight line-broadening is observed during addition of the second equivalent, with the largest effect at ca. 1.5 equivalents of added calcium. These observations indicate that binding to site II is in the intermediate exchange regime. The binding constant $K_{II,I}$ (cf. Fig. 1 for definitions of binding constants) was extracted from computer fits to the chemical shift of well-resolved signals as a function of total calcium. The chemical shift, δ_{calc}, at each titration point was calculated as

$$\delta_{calc} = p \cdot \delta_{Ca2} + (1 - p) \cdot \delta_{Ca1},$$

where δ_{Ca2} and δ_{Ca1} are the chemical shifts in the $(Ca^{2+})_2$ and $(Ca^{2+})_1$ forms, respectively, and p is the fraction of protein in the $(Ca^{2+})_2$ form. p is given by the total protein concentration (calculated from the initial protein concentration and the dilution due to calcium additions), total calcium concentration (cal-

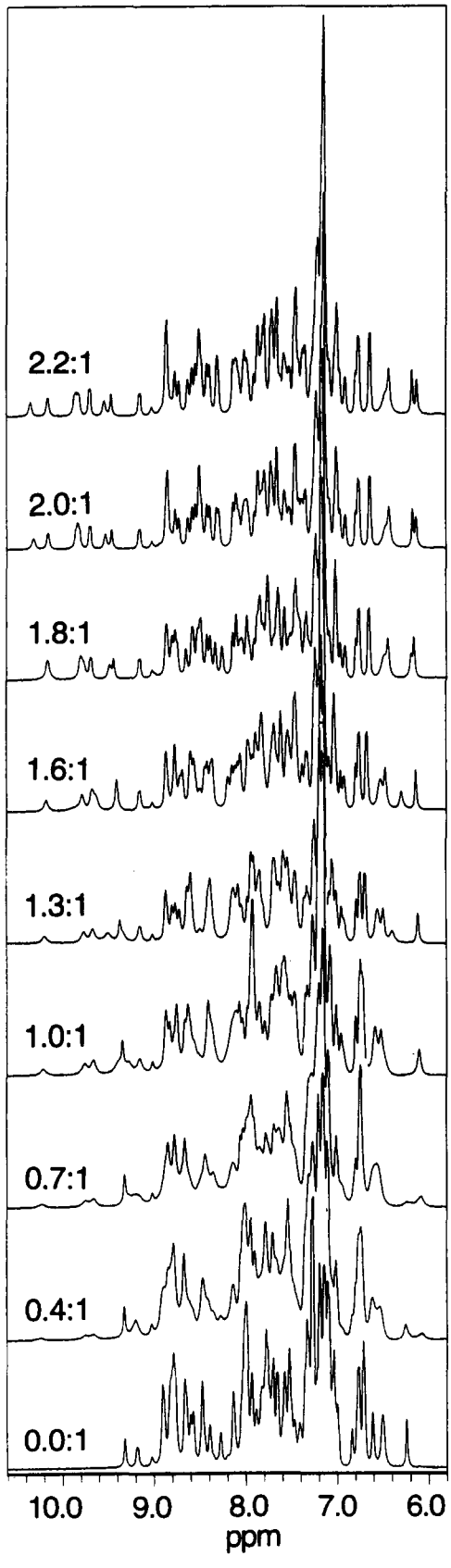

Fig. 2. Sample ^{1}H NMR spectra from a Ca^{2+} titration of the N56A mutant, showing the region of aromatic and amide protons. The Ca^{2+} to protein ratio is indicated for each spectrum.

culated from the calcium additions and dilutions), and the equilibrium binding constant $K_{II,I}$. δ_{Ca2}, δ_{Ca1}, and $K_{II,I}$ were free parameters optimized to obtain the best fit to the measured chemical shifts. All points in the titration were given equal weights. An average of the results from fits to three different signals (at 6.1, 9.5, and 9.8 ppm in the $(Ca^{2+})_2$ protein at pH 7.5) gives a value of $\log K_{II,I} = 4.8 \pm 0.1$. There is a slight overlap of the two binding processes, indicating that Ca^{2+} binding to N56A is not perfectly sequential.

In an effort to closely match the conditions under which the detailed NMR analysis was carried out (see accompanying paper, Wimberly et al., 1995), the concentration of protein in the binding measurements was in the range of 1–2 mM. To rule out any unusual effects associated with these relatively high protein concentrations, a second Ca^{2+} titration was performed at a protein concentration of 30 μM N56A. These binding constant determinations were carried out in the presence of 25 μM of the chelator 5,5'Br_2BAPTA and 2 mM Tris/HCl at pH 7.5. Ca^{2+} was added in steps of 6 μM (from a 3 mM $CaCl_2$ stock solution), and Ca^{2+} binding to the chelator was monitored by recording the absorbance at 263 nm. Computer fits to the absorbance as a function of total Ca^{2+} concentration yielded (as an average of three individual titrations) values of $\log K_1 = 7.1 \pm 0.1$ and $\log K_2 = 5.6 \pm 0.1$.

La^{3+} binding in the absence of Ca^{2+}

The N56A mutant was titrated with $La(ClO_4)_3$ in steps of ca. 0.15 equivalents, and one-dimensional ^{1}H NMR spectra were recorded at each titration point (Fig. 3). These results showed that La^{3+} has the same site preference as observed for wild-type calbindin D_{9k} (Hoffman et al., 1988). At La^{3+} additions between zero and one equivalent, a slow exchange process was observed, which corresponds to binding at site II. There was significant broadening of the lines, with a maximum linewidth, W, of 30 Hz at ca. 0.5 La^{3+} equivalents. The La^{3+} off-rate (k_{off}) from site II, when site I is empty, can be calculated from the maximum linewidth using the following formula, which is valid for slow exchange (Sandström, 1982):

$$k_{off} = \pi W - 1/T_2. \quad (1)$$

If we set $1/T_2 \leq 10$ Hz, which corresponds to the linewidth of the resonances in the absence of exchange, and take into account a contribution to W from scalar coupling as large as 10 Hz, a lower limit of $k_{off} \geq 54$ s^{-1} is obtained. At La^{3+} additions above one equivalent a fast exchange process was observed, corresponding to La^{3+} binding at site I.

Cd^{2+} binding in the absence of Ca^{2+}

Starting with a solution of apo N56A, $Cd(NO_3)_2$ was titrated in steps of ca. 0.14 equivalents, and one-dimensional ^{1}H NMR spectra were recorded at each titration point. Several resonances experienced an upfield or downfield shift when Cd^{2+} was added, but it was not possible to resolve two binding processes. A typical example is shown in Figure 4A (open circles) for the resonance that titrates from 8.84 ppm in the apo state to 9.62 ppm in the $(Cd^{2+})_2$ state. There was also observable broadening of many resonances, with maximum linewidth occurring at 1.0 equivalent of Cd^{2+} (Fig. 4B, open circles). For all resonances,

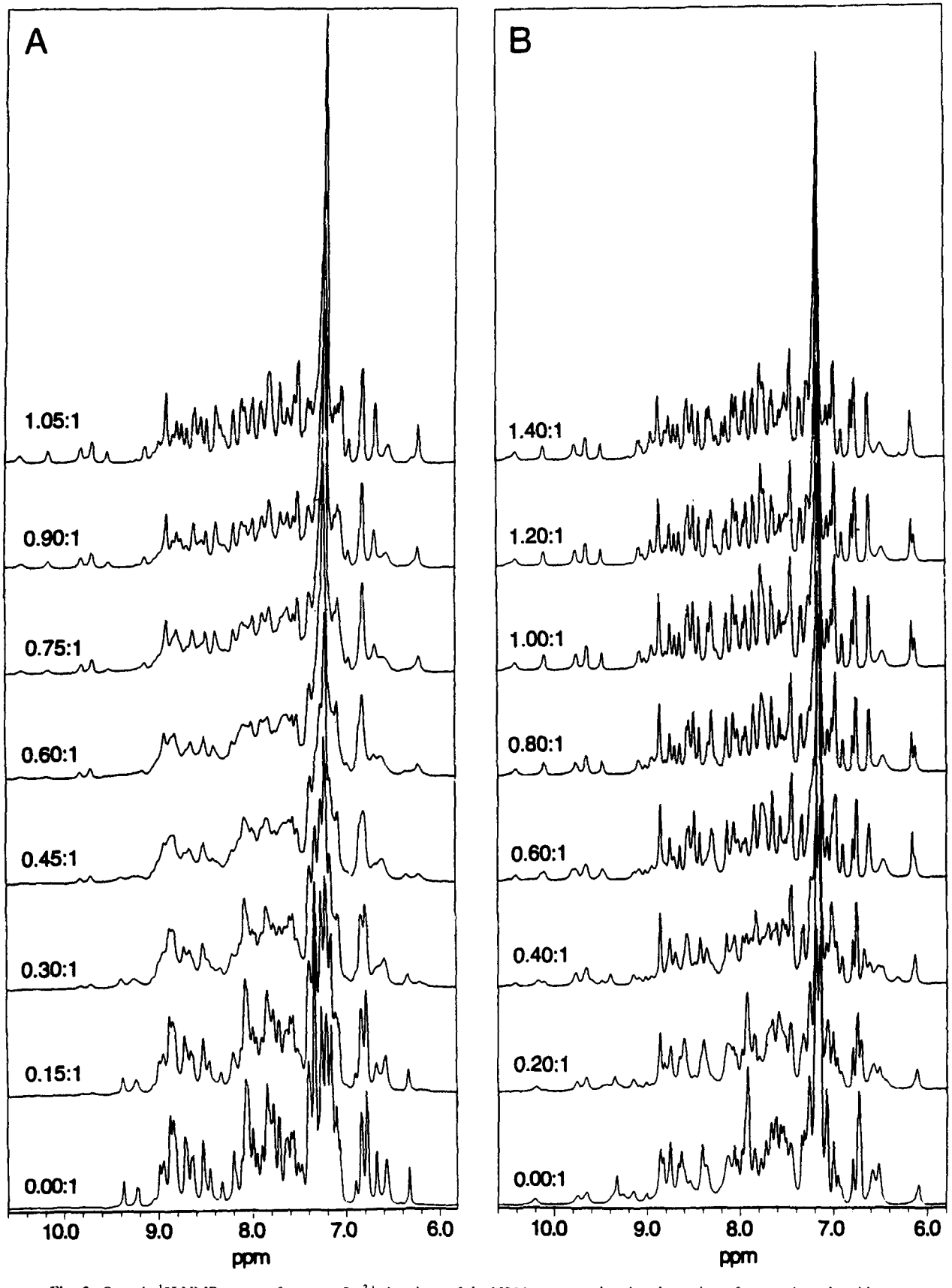

Fig. 3. Sample ^{1}H NMR spectra from two La^{3+} titrations of the N56A mutant, showing the region of aromatic and amide protons. The La^{3+} to protein ratio is indicated for each spectrum. **A:** Titration in the absence of Ca^{2+}. **B:** Titration in the presence of a Ca^{2+} ion in site I.

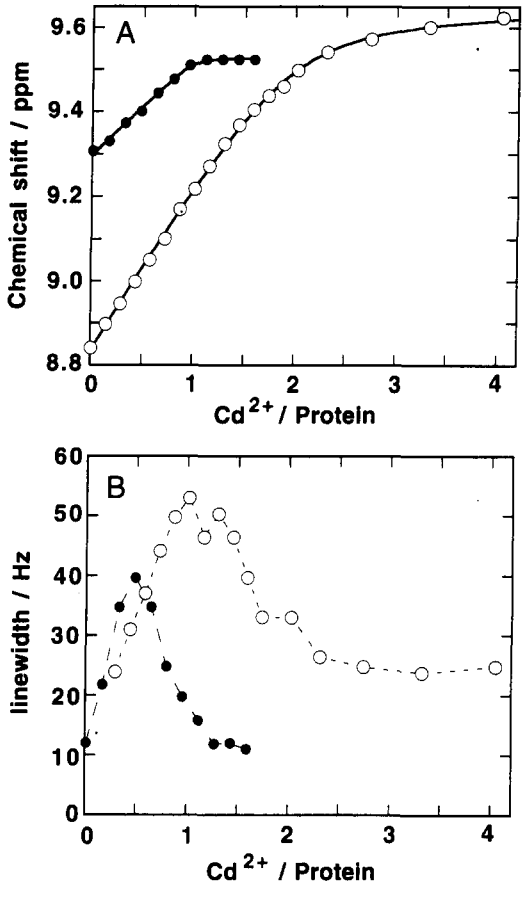

Fig. 4. ^{1}H NMR parameters from two Cd^{2+} titrations of the N56A mutant in the absence (O) and presence (●) of Ca^{2+} in site I. **A:** Chemical shift versus Cd^{2+} to protein ratio. Solid curves were obtained by computer fits to the chemical shift as a function of total Cd^{2+} concentration as described in the text. **B:** Linewidth as function of the Cd^{2+} to protein ratio for the same signals as shown in A.

the chemical shift as a function of total Cd^{2+} concentration reveals only a single binding process, which starts at zero and ends at slightly more than two equivalents of Cd^{2+}. Consequently, the two sites bind cadmium with similar affinities. Computer fits to the chemical shifts of four individual resonances (at 8.7, 8.8, 9.2, and 9.3 ppm in the apo protein) as a function of total Cd^{2+} concentration yield an average binding constant of $\log K = 3.8 \pm 0.1$. Each fit was performed as described above for Ca^{2+} binding using the function

$$\delta_{calc} = p \cdot \delta_{Cd2} + (1 - p) \cdot \delta_{apo},$$

where δ_{Cd2} and δ_{apo} are the chemical shifts in the $(Cd^{2+})_2$ and apo forms, respectively, and p is the fraction of sites that have bound Cd^{2+}. The binding process is in the intermediate exchange regime and an average Cd^{2+} off-rate can be calculated using the following formula (Sandström, 1982):

$$k_{off} = \frac{\pi \delta\nu^2 (W^* + W_0)[1 + 2(W^*/\delta\nu)^2 - (W^*/\delta\nu)^4]^{1/2}}{2(W^{*2} - W_0^2)}, \quad (2)$$

where W^* is the maximum linewidth, W_0 is the linewidth in the absence of exchange, and $\delta\nu$ is the chemical shift difference between Cd^{2+}-free and Cd^{2+}-loaded states. For the well-resolved resonance that titrates from 8.84 ppm in the apo state to 9.62 ppm in the $(Cd^{2+})_2$ state, we find $W^* = 53$ Hz, $W_0 = 20$ Hz, and $\delta\nu = 391$ Hz (cf. Fig. 4). The values for the resonance that titrates from 9.21 to 9.87 ppm are $W^* = 45$ Hz, $W_0 = 22$ Hz, and $\delta\nu = 333$ Hz, and for the resonance that titrates from 8.7 to 10.27 ppm, $W^* = 120$ Hz, $W_0 = 20$ Hz, and $\delta\nu = 785$ Hz. Before calculating k_{off}, a 4–10-Hz contribution from scalar coupling must be subtracted from W^* and W_0, adding 100 s^{-1} to the uncertainty in the value of k_{off}. Thus, the Cd^{2+} off-rate is 8,200 ± 1,200 s^{-1} for N56A.

La^{3+} and Cd^{2+} binding to site II in the presence of Ca^{2+} in site I

In order to measure the La^{3+} and Cd^{2+} binding properties of site II in the presence of a Ca^{2+} ion in site I, calcium was slowly titrated into a stock solution of apo N56A and ^{1}H NMR spectra were recorded to monitor the titration and establish the point at which 1.05 equivalents of calcium were bound. Detection of the endpoint was facilitated by the fact that the first equivalent of calcium binds to site I in slow exchange, whereas binding of the second equivalent gives rise to shifting of resonances (cf. above). Half of this $(Ca^{2+})_1 \cdot$N56A sample was then titrated with La^{3+} in steps of ca. 0.20 equivalents, and the other half was titrated with Cd^{2+}.

A slow exchange process was observed in the La^{3+} titration at additions between zero and one equivalent of lanthanum, with no significant line-broadening (Fig. 3). No further La^{3+} binding was observed at additions above one equivalent. Thus, when a calcium ion is present in site I, the La^{3+} off-rate in site II is ca. 6 s^{-1} or lower ($k_{off} \leq 6$ s^{-1}). This is substantially lower than the value observed for the same site in the absence of calcium ($k_{off} \geq 54$ s^{-1}).

The second half of the $(Ca^{2+})_1 \cdot$N56A sample was titrated with Cd^{2+} in steps of ca. 0.17 equivalents. In this case, a binding process in the intermediate exchange regime was observed at Cd^{2+} additions between zero and one equivalent. No further binding event was observed at additions above one equivalent. Computer fits to chemical shifts of resolved resonances (at 9.31 and 6.70 ppm in the $(Ca^{2+})_1$-form) as a function of total Cd^{2+} concentration yielded an average value of $\log K = 4.9 \pm 0.2$ for the Cd^{2+} binding constant of site II in the presence of a calcium ion in site I. The fitting was performed as described above for Ca^{2+} binding using the function

$$\delta_{calc} = p \cdot \delta_{CaICdI} + (1 - p) \cdot \delta_{CaI},$$

where δ_{CaICdI} and δ_{CaI} are the chemical shifts in the $(Ca^{2+})_1 \cdot (Cd^{2+})_1$ and $(Ca^{2+})_1$ forms, respectively, and p is the fraction of protein that has bound Cd^{2+}. Thus, a significant increase in affinity is observed relative to the average Cd^{2+} binding constant in the absence of calcium (3.8 ± 0.1). The Cd^{2+} off-rate in site II when calcium is bound in site I can be calculated using Equation 2. For the well-resolved resonance at 9.31 ppm in the $(Ca^{2+})_1$ state that is used as an illustration in Figure 4, $W^* = 40$ Hz, $W_0 = 12$ Hz, and $\delta\nu = 108$ Hz. For the resonance at 6.70 ppm, $W^* = 20$ Hz, $W_0 = 12$ Hz, and $\delta\nu = 55$ Hz. As above, a contribution to W^* and W_0 of 4–10 Hz from scalar

coupling must be factored into the analysis, which adds 15 s^{-1} to the uncertainty in the value of k_{off}. Thus, the Cd^{2+} off-rate from site II, with Ca^{2+} bound in site I, is 675 ± 40 s^{-1}, considerably less than the value of k_{off} obtained in the absence of Ca^{2+} (8,200 ± 1,200 s^{-1}). Again, the absence or presence of calcium ion in site I is found to have a profound effect on the off-rate in site II.

Discussion

Cooperativity is a free energy coupling between binding events at separate sites for the same type of ligand. There is no a priori requirement that a cooperative system has two sites with identical affinities. Rather, positive cooperativity is defined as enhancement of ligand affinity at one site associated with the presence of a ligand in the other site. The present work on the N56A calbindin D_{9k} mutant, which binds two calcium ions in a sequential manner, clearly demonstrates that calcium binding at site I has a profound effect on the ion binding characteristics of site II, both in terms of affinity and metal-ion dissociation rates. The variations in site preferences among different metal ions were utilized to circumvent the problem of experimental verification when Ca^{2+} alone is used. We observe that Ca^{2+} binding at site I has a similar effect on site II in terms of both La^{3+} and Cd^{2+} binding. Because cooperativity is a property of symmetry (cf. Fig. 1), it follows that lanthanum or cadmium binding at site II alters the calcium binding properties of site I. Thus, the present data also tell us that the cooperative mechanism in calbindin D_{9k} is not exclusive for calcium ions. Furthermore, the results on binding kinetics suggest that the cooperative mechanism involves a reduction in the metal-ion dissociation rates.

From the observed Cd^{2+} affinity enhancement at site II due to Ca^{2+} binding at site I, we can estimate a value for the free energy coupling between the two binding events, $\Delta\Delta G$, defined as

$$\Delta\Delta G = -RT\ln(K_{II(Cd),I(Ca)}/K_{II(Cd)}). \quad (3)$$

$K_{II(Cd),I(Ca)}$ was measured from the Cd^{2+} titration after one Ca^{2+} equivalent was already bound at site I ($\log K_{II(Cd),I(Ca)} = 4.9 \pm 0.2$). However, in the absence of Ca^{2+}, we found that both sites have similar Cd^{2+} affinities. Thus, only the average binding constant, K ($\log K = 3.8 \pm 0.1$), could be measured, corresponding to the geometric mean of the two macroscopic binding constants (K_1 and K_2) or of two site binding constants (e.g., $K_{II(Cd)}$ and $K_{I(Cd),II(Cd)}$). Because Ca^{2+} binding enhances Cd^{2+} affinity at site II more than 10-fold, it necessarily follows that Cd^{2+} binding at site II enhances the Ca^{2+} affinity at site I more than 10-fold. This suggests that Cd^{2+} binding to N56A is also a cooperative process, and consequently, because the sites have roughly equal Cd^{2+} affinities, $\log K_{II(Cd)} < 3.8$. Using the values determined for the Cd^{2+} binding constant in the presence and absence of Ca^{2+} in site I, the limiting value for the free energy coupling as defined in Equation 3 is $\Delta\Delta G < -6.4$ kJ·mol^{-1}. By comparing to the value of the free energy coupling between Ca^{2+} binding events at the two sites in wild-type calbindin D_{9k} ($\Delta\Delta G = -7.7 \pm 1.7$ kJ·mol^{-1}; Linse et al., 1991), we can conclude that the N56A mutation does not perturb the cooperativity of Ca^{2+} binding to calbindin D_{9k}.

The results of the present work illustrate that in order to derive the molecular basis for positive cooperativity, it is important to recall the strict definition of the phenomenon: binding at one site enhances the affinity at another site. The free energy coupling (or affinity enhancement) between binding events at different sites has a direct physical connection to the binding process. If instead a macroscopic definition of cooperativity in terms of the popular Hill coefficient (Hill, 1910) was used, the N56A mutant would have been categorized as noncooperative because the second macroscopic calcium binding constant is significantly lower than the first ($K_2 < (1/4)K_1$). However, the above results show that calcium binding at one site in N56A enhances the metal-ion affinity for the other site more than 10-fold.

There is an obvious risk when introducing a mutation that is designed to reduce the affinity of a specific site, i.e., the free energy coupling between the binding sites could be perturbed. For mutants designed to bind sequentially and enable study of the cooperative mechanism, it is therefore absolutely essential to establish that the system retains cooperativity. For N56A, it has been shown that the high degree of coupling between the binding sites is conserved, thereby justifying its use as a model for the site I $(Ca^{2+})_I$ state of wild-type calbindin D_{9k} (Wimberly et al., 1995). In addition, the evidence showing that Cd^{2+} binding at one site enhances the metal ion affinity of the other site justifies the use of the wild-type calbindin D_{9k} with one cadmium ion occupying site II as a model for the site II $(Ca^{2+})_{II}$ state of wild-type calbindin D_{9k} (Akke et al., 1991, 1993, 1995).

A final comment is in order regarding the rather large discrepancy between the value obtained for the calcium binding constant $K_{II,I}$ ($\approx K_2$) using ^{1}H NMR at 2 mM protein ($\log K_{II,I} = 4.8 \pm 0.1$) and the value obtained with the chelator method at 30 μM protein ($\log K_2 = 5.6 \pm 0.1$). At first glance this finding might seem highly suspect; however, this observation is fully consistent with the notion that a charged protein can screen electrostatic interactions, as initially predicted by Monte Carlo simulations (Svensson et al., 1991). Such a screening effect would be expected to increase with increasing protein concentration, thus the value measured for ion binding constants will depend on the concentration of protein in solution. We have reported elsewhere on a series of Ca^{2+} titrations of N56A monitored by ^{1}H NMR at protein concentrations ranging from 25 μM to 7 mM, which revealed significant differences in the measured binding constants (Linse et al., 1995). The observed changes in the binding constants were in very good agreement with the values from the corresponding Monte Carlo simulations.

Conclusions

The N56A mutant of calbindin D_{9k} binds metal ions with positive cooperativity, although the sites have widely different affinities. This clearly illustrates that the concepts of sequential and cooperative binding are not mutually exclusive, a conclusion that can be drawn only if using a strict definition of cooperativity as a free energy coupling between binding sites. The data confirm that the $(Ca^{2+})_I$[II] state of N56A and the $(Cd^{2+})_I$[II] state of the wild-type protein are viable models for the two half-saturated states along the corresponding binding pathways to the fully calcium-loaded state. The results also suggest that the cooperative mechanism involves a reduction in the metal-ion dissociation rates.

Materials and methods

The gene for the N56A mutant was constructed as for wild-type calbindin D_{9k} (Brodin et al., 1986) but with the codon for Asp 56 replaced by an alanine codon. The protein was produced in *Escherichia coli* and purified as described (Johansson et al., 1990). 5,5′Br_2BAPTA was purchased from Molecular Probes, Inc. (Eugene, Oregon). All other chemicals were of the highest grade commercially available.

NMR spectra

One-dimensional ^{1}H NMR spectra were recorded at 500.13 MHz on a GE Omega-500 spectrometer, using a presaturation pulse on the solvent signal. The protein concentration was in the range 1–2 mM, dissolved in 2 mM Tris/HCl buffer at pH 7.5 with 10% D_2O. Sixty-four transients were accumulated for each spectrum. One test titration was accompanied by measuring the pH after each metal ion addition to confirm that the buffer concentration was sufficient to prevent pH drift during the titration. Linewidth was measured as the width of the resonance at half-height.

Chelator method

Determinations of calcium binding constants from titrations in the presence of a chromophoric chelator were made as described previously (Linse et al., 1991, 1993).

Acknowledgments

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