A flow-dialysis method for obtaining relative measures of association constants in calmodulin–metal-ion systems

Jeanne M. BUCCIGROSS, Carey L. O'DONNELL and Donald J. NELSON*

Department of Chemistry, Sackler Sciences Center, Clark University, Worcester, MA 01610, U.S.A.

A flow-dialysis apparatus suitable for the study of high-affinity metal-binding proteins has been utilized to study calmodulin–metal exchange as a measure of relative calmodulin–metal association constants. Calmodulin labelled with radioactive ^{153}Gd was dialysed against buffer containing various competing metal ions. The rate of label exchange was monitored by a γ -ray scintillation detector. Competing metals used were Ca^{2+} and Cd^{2+} , and the lanthanides Gd^{3+} , Eu^{3+} , La^{3+} and Lu^{3+} . All exchange processes were first-order, and two categories of metal were found: Ca^{2+} and Cd^{2+} in one, the lanthanides comprising the other. In addition calmodulin–metal complexes with radioactive ^{109}Cd and ^{45}Ca released the bound label without any competing metal being added to the buffer. The kinetics of this metal loss can be described by two consecutive first-order processes, and the fraction of label associated with each rate can be determined. Studies of phosphodiesterase activation by calmodulin show Cd^{2+} and calmodulin to cause 80% of the maximum activation found when Ca^{2+} and calmodulin are used.

INTRODUCTION

Calmodulin (CaM) is a small (M_r 16700), acidic, high-affinity Ca^{2+} -binding protein. Its role as a regulator of Ca^{2+} effects in eukaryotic cells is now well established (Cheung, 1980, 1982; Klee *et al.*, 1980). X-ray-crystallographic data to the 0.3 nm (3 Å) resolution level have recently been published (Babu *et al.*, 1985), which shows that the CaM metal-ion binding sites are the 'EF hand' sites typical of high-affinity Ca^{2+} -binding proteins. In these 'EF hand' sites, the metal-binding loop is flanked by α -helical segments to produce a helix–loop–helix structure. In the loops, the metal ligands are oxygen from side chains and the peptide backbone. CaM has four such sites, with non-equivalent affinities for Ca^{2+} . Although the X-ray structure cannot yet resolve the type of co-ordination in CaM-binding sites (Babu *et al.*, 1985), CaM displays sequence homology with parvalbumin, and the 'EF hands' in this protein show octahedral co-ordination. The same is to be expected for CaM (Kretsinger, 1980).

Numerous studies have been undertaken to determine the dissociation constant, K_d , of these metal-binding sites by a variety of techniques, including equilibrium dialysis (Lin *et al.*, 1979; Watterson *et al.*, 1976; Wolff *et al.*, 1977; Crouch & Klee, 1980; Cox *et al.*, 1981), fluorescence (Kilhoffer *et al.*, 1980a,b), n.m.r. (Seamon, 1980; Forsén *et al.*, 1980), Raman spectroscopy (Seaton *et al.*, 1983) and computer simulations (Delville *et al.*, 1985), but there is little agreement about the magnitude of the binding constants. Most researchers do agree, however, that there are two high-affinity and two low-affinity binding sites on the protein. Recent studies indicate that the sites are filled sequentially rather than randomly (Haich *et al.*, 1981).

The lanthanides have long been recognized as useful probes for the study of Ca^{2+} -binding proteins (Martin,

1983). Metal exchange of the Ca^{2+} -binding protein parvalbumin labelled with Tb^{3+} has been studied by flow dialysis in this laboratory (Gonzalez-Fernandez & Nelson, 1982). The rate of lanthanide exchange caused by competing metal ions was monitored by a γ -ray scintillation detector. In the present study, similar experiments were performed with CaM. Recently ^{113}Cd n.m.r. has also been used to study CaM (Forsén *et al.*, 1980) and so flow-dialysis experiments using radioactive ^{109}Cd were also performed. ^{45}Ca -labelled protein was studied with this technique for comparison purposes. The ability of these various CaM–metal complexes to activate PDE was investigated as a further indication of their usefulness as Ca^{2+} probes.

MATERIALS AND METHODS

Chemicals

Radioactive isotopes (^{153}Gd , 1 mCi; ^{109}Cd , 250 μCi ; ^{45}Ca , 5 mCi) were purchased from New England Nuclear, Bedford, MA, U.S.A., and diluted with 4 ml of water before use, with the exception of ^{45}Ca , which was diluted to 1 ml. All other chemicals were reagent grade. Distilled deionized water was used exclusively. Activator-deficient PDE, 5'-nucleotidase and cyclic AMP were purchased from Sigma, St. Louis, MO, U.S.A.

Calmodulin purification

CaM was purified by the method of Gopalakrishna & Anderson (1982). Fractions (5 ml) were collected and monitored at 230 nm for protein content. CaM-containing fractions were identified by the characteristic phenylalanine fine structure in the region from 280 to 250 nm (Wolff *et al.*, 1977). CaM fractions were combined, freeze-dried from 0.1 M- NH_4HCO_3 and stored desiccated at -80°C until needed. Identity was confirmed by

Abbreviations used: CaM, calmodulin; PDE, 3',5'-cyclic nucleotide phosphodiesterase.

* To whom correspondence and requests for reprints should be sent.

Fig. 1. Schematic diagram of the flow-dialysis apparatus

Abbreviations used: C/T, counter timer; SCA, single-channel analyser; AMP, amplifier; PRE, pre-amplifier; HV, high-voltage supply; PMT, photomultiplier tube; DC, dialysis cell.

^1H n.m.r. and purity was established by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Labelling with radioactive metals

^{153}Gd labelling. All steps were performed at 4°C unless otherwise stated. Approx. 2.8 mg of CaM were dissolved in 2–3 ml of piperazine buffer (10 mM-piperazine/20 mM-KCl, pH 6.5) and dialysed against 1 litre of 2.2 mM- GdCl_3 (non-radioactive) for 24–48 h. The Gd^{3+} -saturated protein was then dialysed against 1 litre of the piperazine buffer for 48 h with one change to remove unbound Gd^{3+} . The CaM was then incubated with 2 ml of $^{153}\text{GdCl}_3$ for 48 h.

Finally, the protein was dialysed against piperazine buffer to remove unbound ^{153}Gd . The final protein concentration was $26\text{--}27\ \mu\text{M}$ as determined by A_{276} ($\epsilon_{276}^M = 3740\ \text{M}^{-1}\cdot\text{cm}^{-1}$) (Wolff *et al.*, 1977).

^{109}Cd labelling. This was similar to the procedure for ^{153}Gd , with the following changes. Initial dialysis was against 200 mM- CdCl_2 and was followed by dialysis against 2 litres of the piperazine buffer with three changes. Labelling was done with 0.8 ml of $^{109}\text{CdCl}_2$. The final protein concentration was $34\ \mu\text{M}$.

^{45}Ca labelling. Again, this was similar to the procedure for ^{109}Cd , except labelling was done with 0.2 ml of $^{45}\text{CaCl}_2$. The final protein concentration was $34\ \mu\text{M}$.

Flow dialysis

Flow dialysis was performed as described previously (Gonzalez-Fernandez & Nelson, 1982; Bean *et al.*, 1983), except that FMI laboratory pumps set at a flow rate of 120 ml/min were used rather than peristaltic pumps. The temperature was maintained at $23.3 \pm 1.1^\circ\text{C}$. Briefly, the flow-dialysis cell was composed of two halves of a Lucite (Perspex) block, bolted together and separated only by a dialysis membrane. The top half contained a $1\ \text{cm}^3$ protein compartment and was separated by a dialysis membrane (M_r cut-off 6000–8000) from the lower (buffer) side. The lower half had inlet and outlet tubes to allow for a continuous flow of buffer through the system.

The cells were mounted in front of NaI γ -scintillation detectors, both of which were housed inside a lead cave to reduce background noise (Fig. 1). The output from the detectors was amplified before being directed to single channel analysers. The single-channel analysers were used to select the specific energy radiation of the metal nucleus under investigation and thus further decrease the background noise. This output was fed to a counter/timer. Data accumulated for a dwell time of 2000 s were recorded by a line printer.

Flow dialysis, like all dialysis processes, is an equilibrium process. Clearly then, it cannot be used to measure protein-metal off-rates but only relative protein-metal association constants.

In the case of the ^{109}Cd and ^{45}Ca experiments, spontaneous metal loss was observed and no competing metal was used. With Gd^{3+} a competing metal was required to displace the label. Competing metal-ion concentrations used were $37.5\ \mu\text{M}$ for the lanthanides, except as noted, and 10 mM for Ca^{2+} and Cd^{2+} .

Data analysis

Metal-exchange data for ^{153}Gd were fitted by a least-squares program to a first-order decay of the form:

$$A_t = (A_0 - A_\infty) \exp(-kt) + A_\infty \quad (1)$$

Where A_t is the value of 'counts/2000s' at any time, t , A_0 is the initial value and A_∞ is the value at the end of the reaction. A_0 , A_∞ and k are all least-squares parameters.

The ^{109}Cd and ^{45}Ca data were fitted to a least-squares program (Wentworth, 1965) for two consecutive first-order processes of the form:

$$A_t = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_\infty \quad (2)$$

where k_1 and k_2 are the rate constants for the two processes, A_1 , A_2 are amplitudes of each process, and A_t and A_∞ are defined as above. All five are least-squares parameters.

In all cases calculated and observed values for A_0 and

Fig. 2. Exchange of CaM-bound ^{153}Gd with competing non-radioactive Gd^{3+} at 0.05 mM concentration

The continuous line represents the theoretical curve.

A_{∞} were in good agreement. Results presented are the average of two runs and were reproducible within $\pm 20\%$, as indicated.

PDE activation by CaM

CaM activation of PDE was studied by the two-step method of Butcher & Sutherland (1962) as modified by Teo & Wang (1973) with slight changes. The P_i content was determined by the method of Sanui (1974). Briefly, the reaction buffer was 40 mM-Tris-HCl/40 mM-imidazole/3 mM-magnesium acetate. Final concentrations of reactants were 0.12 unit of PDE, 0.1 μM -calmodulin, 1.2 mM-cyclic AMP and various metal concentrations from 5.3 μM to 0.53 mM in a volume of 0.9 ml.

The Tris/imidazole buffer was freed of Ca^{2+} by passage through a Chelex column (1.3 cm $\times$ 24 cm) before the addition of magnesium acetate. Magnesium acetate was recrystallized to remove trace Ca^{2+} present. After recrystallization, a 1 M solution contained Ca^{2+} concentrations of less than 1 μM as determined by atomic-absorption spectroscopy. The final concentration of magnesium acetate in the buffer was 3 mM, so the amount of Ca^{2+} contributed is less than 3 nM. CaM and PDE were freed of Ca^{2+} by use of the EGTA-treatment procedure of Teo & Wang (1973).

The PDE reaction was run at 30 $^{\circ}\text{C}$ for 30 min and terminated by placing the tubes in a boiling-water bath for 3 min. Samples were then returned to 30 $^{\circ}\text{C}$ by placing them in the water bath for 6 min. 5'-Nucleotidase (0.02 unit) was added and the tubes were incubated for 15 min. The nucleotidase reaction was terminated by the addition of 4 ml of ice-cold 10% (w/v) trichloroacetic acid, the tubes were then placed in an ice bath for 5 min, and centrifuged for 8 min at 2000 g ($r_{\text{av.}} = 9.30$ cm). A 1 ml aliquot of the supernatant was transferred to a clean dry

Table 1. Rate constants for ^{153}Gd -exchange experiments

Rate constants were calculated from eqn. (1) as described in the text. Two classes of competing metals are clearly observed.

Competing metal	Concn. (mM)	k (h^{-1})
Ca(II)	10	$9.81 \pm 0.35 \times 10^{-3}$
Cd(II)	10	$1.50 \pm 0.18 \times 10^{-2}$
Eu(III)	0.0375	$3.85 \pm 0.32 \times 10^{-2}$
Gd(III)	0.0375	$2.66 \pm 0.01 \times 10^{-2}$
La(III)	0.0375	$1.44 \pm 0.02 \times 10^{-2}$
Lu(III)	0.0375	$1.38 \pm 0.018 \times 10^{-2}$

centrifuge tube for P_i extraction, as phosphomolybdate, into butyl acetate. The u.v. absorbance was monitored at 310 nm (Sanui, 1974). CaM concentration was chosen on the basis of a concentration study (results not shown). Concentrated stock solutions of all reactants were made in the Tris/imidazole buffer. The same stocks were used for all runs to avoid possible errors.

At each metal concentration three controls were run, a basal-activity control (no CaM), a metal-free control and a blank (no phosphodiesterase). In all cases the metal-free activity was equivalent to basal activity found in the absence of CaM. Activities were corrected by subtracting the blank value and normalized to the basal-activity level.

RESULTS

^{153}Gd -protein

^{153}Gd exchange exhibited first-order kinetics when fitted by a least-squares program (Fig. 2). The competing metals fell into two categories, with Ca^{2+} and Cd^{2+} comprising one, and the lanthanides the other (Table 1). In the absence of competing metals, no Gd^{3+} loss was observed, indicating that the Gd^{3+} is very tightly bound to the protein.

Although the rate of Gd^{3+} loss from the cell is measured as a function of the $[\text{Gd}^{3+}]_{\text{total}}$ ($[\text{Gd}^{3+}]_{\text{free}} + [\text{Gd}^{3+}]_{\text{bound}}$), the rate of diffusion from the cell is actually dependent upon the $[\text{Gd}^{3+}]_{\text{free}}$ at any time.

$$\text{Rate} = k_{\text{obs.}}[\text{Gd}^{3+}]_{\text{total}} = k'[\text{Gd}^{3+}]_{\text{free}} \quad (3)$$

Since the metal-exchange process can be described by separate equilibria coupled through the unbound form of the protein, $[\text{Gd}^{3+}]_{\text{free}}$ at any time can be expressed analytically as:

$$[\text{Gd}^{3+}]_{\text{free}} = \frac{K_X [\text{CaM} \cdot \text{Gd}]}{K_{\text{Gd}} [\text{CaM} \cdot \text{X}]} [\text{X}] \quad (4)$$

where K_{Gd} and K_X are association constants for the metal-protein complex and $[\text{X}]$ is the concentration of competing metal. Since the competing metal is present in great excess relative to the protein and Gd^{3+} , its concentration can be considered as constant throughout the experiment. The ratio of $[\text{CaM} \cdot \text{Gd}]/[\text{CaM} \cdot \text{X}]$ is a function only of $[\text{Gd}^{3+}]_{\text{total}}$ at a given $[\text{X}]$. A larger K_X would produce a larger free $[\text{Gd}^{3+}]$ and thus a faster rate. The lanthanides would be expected to have larger association constants, since they are similar in size to

Fig. 3. A plot of rate constant for ^{153}Gd exchange as a function of non-radioactive Gd^{3+} concentration

Fig. 4. A plot of ^{109}Cd experiments showing biphasic metal release from the dialysis cell

The continuous line represents the least-squares fit.

Ca^{2+} , have a 3+ valence state and, like Ca^{2+} , ‘prefer’ co-ordination to oxygen. Since the fit considers the $[\text{Gd}^{3+}]_{\text{total}}$, k_{obs} contains all the terms in eqn. (4) above:

$$k_{\text{obs.}} = \frac{k'}{[\text{Gd}]_{\text{total}}} \frac{K_x}{K_{\text{Gd}}} [\text{X}] f(\text{Gd})_{\text{total}} \quad (5)$$

where $f(\text{Gd})$ indicates that $[\text{CaM} \cdot \text{Gd}]/[\text{CaM} \cdot \text{X}]$ is a function of $[\text{Gd}^{3+}]_{\text{total}}$. From eqn. (5) it is also predicted that $k_{\text{obs.}}$ should be linear with $[\text{X}]$, the competing metal. This was observed experimentally using non-radioactive Gd^{3+} as the competing metal (Fig. 3). The rate constant, k' , was determined by control experiments using ^{153}Gd with no protein in the cell. Concentrations of competing metal were chosen so that k' would not be rate-limiting. In this case, the observed rate constant, $k_{\text{obs.}}$, is a measure of the ratio of association constants of competing metal to Gd^{3+} . The values of $k_{\text{obs.}}$, then, represent relative association constants for the metals studied. It should also be noted that the rate law shows no dependence on $[\text{CaM}]$ and experimental results (not shown) confirm this.

^{45}Ca - and ^{109}Cd -labelled protein

The ^{109}Cd - and ^{45}Ca -labelled proteins did not show simple first-order kinetics (Fig. 4). These processes were fit to the equation for two consecutive first-order processes, to produce two rate constants (Table 2). [A double-exponential fit can also be interpreted as two simultaneous processes. Such an interpretation would change the physical meaning of the constants A_1 and A_2 , but would not affect the calculated values. In this case, consecutive processes were chosen because of the study by Haich *et al.* (1981), which indicated that CaM sites are filled sequentially rather than randomly.]

As with Gd^{3+} , control experiments were performed to determine the rate constant by which free metal leaves the cell (Table 2). This process exhibited simple first-order loss, with a rate constant, k' . Since these rate constants were close to the observed k_1 values, it was necessary to demonstrate that some of the metal loss associated with k_1 was due to bound metal. This was done experimentally in two ways, first by ultrafiltration, and second by comparison of the observed rate constants. In the first method, a 500 μl aliquot of the protein solution was counted without circulating buffer. A 3–5 ml portion of the same protein solution was rapidly ultrafiltered to produce 1–1.5 ml of filtrate, from which a 500 μl aliquot was counted for radioactivity. The concentration of unbound metal in the filtrate must be the same as that in the protein solution, whereas bound metal would be retained with the protein. Comparison of the radioactivity counts from this sample with those from the unfiltered

Table 2. Rate constants for ^{45}Ca and ^{109}Cd experiments

Rate constants k_1 and k_2 are calculated by the best fit to eqn. (2) as described in the text. Control experiments are also described in the text.

Metal	k_1 (h^{-1})	k_2 (h^{-1})	k' control (h^{-1})
^{45}Ca	$1.91 \pm 0.04 \times 10^{-1}$	$1.68 \pm 0.03 \times 10^{-2}$	$2.12 \pm 0.08 \times 10^{-1}$
^{109}Cd	$1.48 \pm 0.13 \times 10^{-1}$	$2.91 \pm 0.5 \times 10^{-2}$	$2.29 \pm 0.08 \times 10^{-1}$

Table 3. Relative amounts of metal-ion associated with each rate process in the ^{45}Ca and ^{109}Cd experiments

Metal		Fraction of total counts associated with each rate constant*	Percentage of k_1 fraction initially unbound	
			Using ultrafiltration	Using $k_{1, \text{obs.}}/k'$
^{45}Ca	k_1	0.534 ± 0.018	79 ± 3	90 ± 4
	k_2	0.466 ± 0.018		
^{109}Cd	k_1	0.68 ± 0.09	62 ± 8	65 ± 6
	k_2	0.32 ± 0.09		

* Determined by use of the least-squares parameters (see eqn. (2)).

Table 4. Gd^{3+} inhibition of PDE

It was not possible to determine whether $\text{Gd} \cdot \text{CaM}$ could activate PDE, since the lanthanide ion directly inhibited PDE.

Metal*	$[\text{P}_1]^\dagger$ (μM)
Gd	2.64
Gd basal	2.42
Ca basal	25.55
Blank	2.86

* 10 nM-CaM, 0.53 mM-metal.

† These values are uncorrected for the blank (see the Materials and methods section).

sample, and the least-squares parameters A_1 , A_2 and A_∞ indicate that, although unbound metal is present, its concentration is not high enough to explain all the loss associated with the fast rate. A significant amount of bound metal is associated with k_1 (Table 3).

As with the Gd-labelled CaM, the observed rate of metal loss is proportional to the concentration of free metal, but is measured as a function of the total metal associated with each rate constant. Since the fraction of free metal is inversely dependent upon the metal-binding constant of the protein, the two different rate constants observed reflect two different metal-affinity constants of CaM and again represent a measure of relative association constants of the protein. A larger binding

constant, as in the high-affinity sites, will result in a smaller observed rate constant. For each rate constant the rate law can be written as:

$$\text{Rate} = k_{\text{obs.}}[\text{M}]_{\text{total}} = k'[\text{M}]_{\text{free}} \quad (6)$$

where $[\text{M}]_{\text{total}}$ represents the total metal associated with the rate constant $k_{\text{obs.}}$, and k' is the control value determined for the loss of free metal from the protein cell.

From eqn. (6) it can be seen that the fraction of free metal at any time is equal to the ratio of the observed rate constant to the rate constant for the loss of free metal ($k_{\text{obs.}}/k'$). This provides the second method to show that some of the metal lost with k_1 is initially bound. From k_1 and k' estimates can be made for the percentage of metal associated with k_1 which is free. These results are shown in Table 3 and are in good agreement with those determined by ultrafiltration. If none of this metal were bound, k_1 would be equal to k' . Comparison of k_2 and k' show that almost all the metal associated with this process is bound at any given time. In addition to experimental results, calculations done using approximate CaM-metal affinity constants and experimental values of protein and metal concentrations, predict free metal to total metal concentrations in agreement with these results.

Activation of PDE

CaM activates PDE in a Ca^{2+} -dependent manner (Cheung, 1980). Studies have shown that other metals can substitute for Ca^{2+} in the CaM-mediated activation of PDE (Teo & Wang, 1973; Cox *et al.*, 1981; Haberman

Table 5. PDE activation by Ca-CaM and Cd-CaM

Cd^{2+} activates PDE to $\sim 80\%$ of the Ca^{2+} -induced maximum. See the text for details.

$-\log [\text{M}^{2+}]$	Relative activity*		Total activity (%)†	
	Ca^{2+}	Cd^{2+}	Ca^{2+}	Cd^{2+}
3.28	4.76 ± 0.11	4.03 ± 0.21	100 ± 4	81 ± 6
3.78	4.50 ± 0.18	4.19 ± 0.08	93 ± 6	85 ± 2
4.28	4.28 ± 0.21	4.00 ± 0.17	80 ± 6	80 ± 5
4.78	1.36 ± 0.06	1.33 ± 0.22	9.3 ± 1.6	9 ± 6
5.28	1.02 ± 0.04	1.04 ± 0.01	0.5 ± 1.1	1.1 ± 0.3

* Relative to CaM-free basal activity.

† This scale is designed such that basal activity equals 0% and the Ca^{2+} -induced maximum equals 100%.

et al., 1983). In the present study, an attempt was made to compare the activating abilities of Gd^{3+} and Cd^{2+} with Ca^{2+} . Ca^{2+} -free buffers and proteins were prepared (see the Materials and methods section). In addition, a metal-free control, a CaM-free control (basal level) and a PDE-free control (reaction blank) were run at each metal concentration. All results are reported as activity above basal to eliminate any possible variations between different runs; one set of stock solutions was used for all runs. Metal-free runs were equivalent to basal activities within experimental error. These controls were necessary to ensure that the 'Ca-free' system did not contain metal contaminants sufficient to cause activation in the absence of added metal.

Gd^{3+} -containing samples tested at various CaM concentrations showed P_i concentrations equivalent within experimental error to the PDE-free blank. This was well below the CaM-free basal activity of the enzyme in the presence of Ca^{2+} . In order to determine whether this PDE inhibition was due to $\text{Gd} \cdot \text{CaM}$ or was a direct effect of Gd^{3+} on the enzyme, the basal level was determined in the presence of Gd^{3+} . This sample also showed inhibited PDE activity, thus indicating a direct inhibition of the enzyme by Gd^{3+} . Due to this direct PDE inhibition, it was not possible to determine the ability of the $\text{Gd} \cdot \text{CaM}$ complex to activate PDE (Table 4).

Cd^{2+} , on the other hand, did cause CaM activation of PDE. As with Gd^{3+} , basal levels were run to ensure that the Cd^{2+} was activating through the CaM. Basal levels in the presence of Cd^{2+} were equivalent to those in the presence of Ca^{2+} , thus indicating $\text{Cd} \cdot \text{CaM}$ activation of PDE.

The maximal activation achieved with Cd^{2+} and CaM was about 80% of that observed with Ca^{2+} and CaM (Table 5). Both Cd^{2+} and Ca^{2+} reached saturation at approximately the same metal concentration; in fact, the results suggests that Cd^{2+} may actually reach saturation at a slightly lower concentration.

DISCUSSION

The flow-dialysis studies clearly show that Cd^{2+} loss and Ca^{2+} loss are more alike than are Ca^{2+} and Gd^{3+} loss. In the case of Ca^{2+} and Cd^{2+} , the two classes of sites are clearly seen and the Ca^{2+} and Cd^{2+} show similar affinities for the protein. Since the two observed rate constants (k_1 and k_2) for both Ca^{2+} and Cd^{2+} are associated with some bound metal, it is clear that they correspond to the two classes of binding sites. The k_{obs} values are a measure of the relative metal binding constants, and so the Cd^{2+} binding constants must be close to those for Ca^{2+} . The rates of $\text{Ca}^{2+} \cdot \text{CaM}$ dissociation, off-rates, induced by EDTA and quin 2 have been measured by using stopped-flow fluorescence, and two rate constants were observed (Bayley *et al.*, 1984). These rate constants were assumed to correspond to the high- and low-affinity binding sites, and support two classes of association constants for Ca^{2+} , as do the numerous binding studies.

The lanthanide-exchange experiments with ^{153}Gd -labelled CaM showed no evidence of biphasic kinetics. Similar results were seen with ^{155}Eu (results not shown). One possibility for this could be that Gd^{3+} binds to all four sites with equal (or near equal) affinity, since the k_{obs} is a measure of relative association constants. Some studies indicate that the environment in all four binding

sites for the lanthanide Eu^{3+} are similar, since the electronic transitions of the metal occur at the same wavelength in all sites (Wang *et al.*, 1982). Recent studies have indicated that the sequence of filling is different with Tb^{3+} than with Ca^{2+} and Cd^{2+} . It appears that sites III and IV are the high-affinity sites for Ca^{2+} and Cd^{2+} , but sites I and II are filled first with the lanthanide Tb^{3+} and are assumed to be high-affinity sites for that metal (Wang *et al.*, 1984). In addition, stopped-flow studies by the same group, have shown different off-rates for $\text{Tb} \cdot \text{CaM}$, suggesting two classes of sites for Tb^{3+} . Since the on-rates were not directly measured, only approximate affinity constants were calculated on the basis of observed off-rates and assumed diffusion-controlled on-rates (Wang *et al.*, 1984).

The lack of biphasic metal exchange seen in the present study cannot be taken as evidence against two classes of lanthanide-binding sites. From eqn. (5) it is clear that, in the case of metal exchange, the k_{obs} depends upon the ratio of affinity constants for both metals. If the difference between high- and low-affinity constants is approximately the same for both metals, the ratio would remain nearly constant and only one rate constant would be observed. This result is consistent with the Gd^{3+} -lanthanide-exchange data.

In the case of Ca^{2+} - and Cd^{2+} -induced exchange of Gd^{3+} , such an occurrence is less expected, in view of the reverse affinities observed with the lanthanide Tb^{3+} and Ca^{2+} (Wang *et al.*, 1984). The present study employed Gd^{3+} , however, and it is entirely possible that Gd^{3+} shows a preference for binding sites different from that shown by Tb^{3+} . Such differences among lanthanides have been observed in the homologous Ca^{2+} -binding protein parvalbumin. In this protein, Ca^{2+} has the same affinity for both the 'CD' and 'EF' sites, as does Gd^{3+} , but other lanthanides show differences in affinity, with some showing higher affinity for the 'CD' site and others showing preference for the 'EF' site (Williams *et al.*, 1984).

The main conclusion from the metal-ion-exchange studies is that all the lanthanides tested have much higher affinity for CaM than do either Ca^{2+} or Cd^{2+} . In these studies, the concentration required for Ca^{2+} and Cd^{2+} as competing metals was over 250 times greater than the concentration of competing lanthanides needed to produce nearly equivalent exchange rates. The rate constants obtained in this manner were very close for Ca^{2+} , Cd^{2+} , La^{3+} and Lu^{3+} , and slightly larger for Gd^{3+} and Eu^{3+} , leading to the conclusion that lanthanide affinity constants should be 250–500 times greater than those observed with Ca^{2+} . By using the reported Ca^{2+} binding constants in the ranges of 3×10^5 – $1 \times 10^6 \text{ M}^{-1}$ for the high-affinity sites and 1×10^3 – $3 \times 10^4 \text{ M}^{-1}$ for the low-affinity sites yields lanthanide constants in the ranges of 7.5×10^7 – $5 \times 10^8 \text{ M}^{-1}$ and 2.5×10^5 – $1.5 \times 10^7 \text{ M}^{-1}$ for the high- and low-affinity sites respectively. These are in reasonable agreement with the approximate values of $1.7 \times 10^7 \text{ M}^{-1}$ and $1.8 \times 10^8 \text{ M}^{-1}$ for the high-affinity sites and $1.5 \times 10^5 \text{ M}^{-1}$ for the low-affinity sites reported by Wang *et al.* (1984).

The activity studies presented here also indicate that Cd^{2+} behaves similarly to Ca^{2+} in its ability to activate phosphodiesterase in the presence of CaM. The results shown here, indicating that Cd^{2+} produces approx. 80% of the maximum activation attainable with Ca^{2+} , are in contrast with the results of a previous study using lower

metal concentrations, which showed Cd^{2+} to be relatively ineffective (Haberman *et al.*, 1983). One difference between their work and the present study is their use of Mn^{2+} as the PDE cofactor rather than the natural Mg^{2+} cofactor used in the present study. The metal-free control rules out the possibility of activation by something other than the added Cd^{2+} . Unfortunately the Gd^{3+} could not be directly compared, owing to its direct inhibition of the PDE (see the Results section).

Recently X-ray-crystallography studies, using EGTA as a model of the CaM binding sites, have shown that crystal structures of the Cd^{2+} -EGTA chelate and the Ca^{2+} -EGTA chelate are very similar. Both metals show the same order of preference for the ligands, as judged by bond distances, and show the same overall geometry. Further results by this group also show that the crystal structures of lanthanide-EGTA chelates are very different (C. K. Schauer & O. P. Anderson, personal communication).

The flow-dialysis data show that Cd^{2+} and Ca^{2+} have similar biphasic behaviour. The magnitudes of the rate constants indicate that Ca^{2+} and Cd^{2+} have similar affinities for CaM, whereas the lanthanides show much higher affinity. In addition, Cd^{2+} is able to activate PDE to 80% of the Ca^{2+} level. These data and the data of C. K. Schauer & O. P. Anderson (personal communication), indicate that Cd^{2+} is a better probe for Ca^{2+} -CaM interactions than the lanthanides. This is of particular importance for ^{113}Cd -n.m.r. experiments.

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APPENDIX

BIPHASIC LOSS OF METAL FROM THE CELL

The occurrence of biphasic metal loss would be expected for any metal ion with two different affinity constants (two classes of sites) for the protein. This can be most easily visualized in the case of consecutive filling (and release) of metal, but is equally valid for simultaneous processes.

First, in the case of obligatory ordered release, the free metal concentration in the cell will depend initially upon the low-affinity sites only. Since this affinity constant is low, the free metal concentration will be high, and metal loss from the cell rapid (corresponding to a large k). As this occurs, the CaM species with metal only in the

high-affinity sites will be produced. The equilibrium of this species will result in a lower free metal/bound metal ratio than the low-affinity sites. Thus, as the low-affinity sites are emptied, the concentration of free metal in the cell will depend upon the high-affinity constant, and will decrease. Consequently, the rate of metal loss from the cell, which depends on free metal concentration, will also decrease (corresponding to a smaller k). These two processes are mathematically resolved by eqn. (2) of the main paper.

In the case of simultaneous metal release from the protein, a biphasic curve would still be seen. The initial

free metal concentration inside the cell will depend upon the affinity constants for both classes of sites (low and high affinity). Clearly the free metal/bound metal ratio will be higher for the low-affinity sites. In fact the relatively large amount of free metal released from these sites will tend to keep the metal in high-affinity sites bound, so that most of the free metal initially present will be from low-affinity sites. The rate of metal loss from the cell will be fast, corresponding to a large k .

As this continues, eventually most of the metal will be gone from the low-affinity sites, while much of the metal

will still be remaining in the high-affinity sites. This now means that the free metal concentration in the cell at this time, will be determined primarily by the high-affinity constant, and thus will be lower than before. The lower free metal concentration will result in a slower rate of metal loss from the cell, and thus a smaller k . At times between these two limits, the concentration of free metal will be determined by both constants. Once again these two processes can be resolved mathematically by eqn. (2) of the main paper.