Shields, D., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2059–2063.
Wetmore, A. (1960) *Smithson. Misc. Collect. 139*, 1–37.
White, T. J., Mross, G. A., Osserman, E. F., & Wilson, A. C. (1977) *Biochemistry 16*, 1430–1436.
White, T. J., Ibrahimi, I. M., & Wilson, A. C. (1978) *Nature (London) 274*, 92–94.
Wilson, A. C., & Prager, E. M. (1974) in *Lysozyme* (Osserman, E. F., Canfield, R. E., & Beychok, S., Eds.) pp 127–141, Academic Press, New York.

Magnesium and Calcium Binding to Parvalbumins: Evidence for Differences between Parvalbumins and an Explanation of Their Relaxing Function†

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ABSTRACT: The calcium- and magnesium-binding properties of four parvalbumins, two from each genetic lineage α and β, have been studied by equilibrium dialysis, flow dialysis, and ultraviolet differential spectroscopy. In the absence of calcium, α-parvalbumins from rabbit pI = 5.55 and from frog pI = 4.88 and β-parvalbumin from frog pI = 4.50 bind two Mg^{2+} ions with high affinity (K_d = 16, 21, and 27 μM, respectively) at two equivalent and independent sites. Magnesium binding to the ion-free proteins induces conformational changes at the level of the hydrophobic core and of the AB loop, which can be monitored by UV differential spectroscopy. Absorbance differences increase linearly with the molar ratio up to 2 mol of Mg^{2+}/mol of parvalbumin. The affinity of parvalbumins for Ca^{2+} decreases in the presence of Mg^{2+} according to a simple competition for the same sites. In the absence of Mg^{2+}, two Ca^{2+} ions are bound at equivalent and independent sites with a calculated K_d = 6.6, 7.8, and 2.2 nM for rabbit, frog pI = 4.88, and frog pI = 4.50 parvalbumins, respectively. Substitution of Mg^{2+} by Ca^{2+} ions induces structural changes that are especially visible at the level of the hydrophobic core and of the AB loop, but the overall structure is similar in proteins that bind either Ca^{2+} or Mg^{2+}. Therefore, these three parvalbumins exhibit two independent and equivalent high-affinity Ca^{2+}–Mg^{2+} sites. In contrast, when studied by the above techniques, β-parvalbumin from hake exhibits two nonequivalent high-affinity Ca^{2+}–Mg^{2+} sites. Since no more than 1.5 Mg^{2+}- or Ca^{2+}-binding sites could be found, the protein may have lost part of its Ca^{2+}-binding capacity upon removal of divalent metals. Hake parvalbumin was shown to bind 2 Ca^{2+} ions at independent sites with K_{d_1} = 3–5 nM and $K_{d_2} \geq$ 17 nM, when conformational changes induced by addition of EGTA were followed by UV differential spectroscopy. Binding of EGTA to parvalbumin ($K_{0.5}$ = 35 mM) was also demonstrated by this technique. β-Parvalbumins appear to bind Ca^{2+} ions more strongly than α-parvalbumins. The Ca^{2+}–Mg^{2+} sites of parvalbumins exhibit either a lysyl residue (CD sites) or a glycyl residue (EF sites) between the Y and Z coordination sites. There is, therefore, no correlation between the nature of the residue in this position and the class of site. Parvalbumins bind 2 Mg^{2+} ions in resting muscle. The delay in calcium binding resulting from the dissociation of bound Mg^{2+} explains why Ca^{2+} can trigger contraction. Relaxation occurs when Ca^{2+} is displaced from the low-affinity sites of troponin C to the high-affinity Ca^{2+}–Mg^{2+} sites of parvalbumins. More generally, low-affinity Ca^{2+}-specific sites, which pick up Ca^{2+} with diffusion-limited kinetics, are "triggering sites", whereas high-affinity Ca^{2+}–Mg^{2+} sites are "relaxing sites".

Parvalbumins are low M_r (ca. 12000) acidic proteins present in abundance in the sarcoplasm of vertebrate fast skeletal muscles (Pechère et al., 1973; Blum et al., 1977; Pechère, 1977). They belong to two evolutionarily distinct lineages α and β, which were recognized in phylogenetic trees built by the maximum parsimony method (Goodman & Pechère, 1977) and confirmed by the absence of immunological cross-reactions between α and β proteins (Demaille et al., 1974). The knowledge of a number of primary structures (see Goodman & Pechère, 1977, for a review) and of the tertiary structure (Moews & Kretsinger, 1975a) of parvalbumins is in sharp contrast with the fact that their biological function is as yet poorly understood. There is some evidence, however (Pechère, 1977), that this function is related to their calcium-binding properties. By using the Chelex partition technique, parvalbumins were shown (Benzonana et al., 1972) to bind 2 mol of Ca^{2+}/mol with a $K_d = 2 \times 10^{-7}$ M in the presence of physiological levels of Mg^{2+}, i.e., 2 mM. They are mostly found in fast muscle and nervous tissue (Baron et al., 1975), which both possess Ca^{2+} sequestration devices such as sarcoplasmic reticulum, allowing fast triggering and release (Hasselbach, 1978; Blaustein et al., 1978).

It was thus recently proposed that parvalbumins play the role of a soluble relaxing factor, capable of removing Ca^{2+} from the myofibrillar troponin C before being themselves deionized by the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}–ATPase (Pechère et al., 1977; Pechère, 1977). This hypothesis has received experimental support from kinetic experiments conducted on myofibrils (Pechère et al., 1977) as well as from the ability of sarcoplasmic reticulum vesicles to remove Ca^{2+} from parvalbumins (Blum et al., 1977; Gerday & Gillis, 1976).

Parvalbumins must therefore exist in resting muscle in the Ca^{2+}-free form. This raises the question as to how calcium ions can reach troponin C through the parvalbumin "barrier"

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at the onset of contraction when Ca^{2+} is released from the sarcoplasmic reticulum. The concentration of parvalbumin is indeed very high in fast muscle fibers (Blum et al., 1977; Baron et al., 1975) and their affinity for Ca^{2+} is ca. one order of magnitude higher than the one of troponin C (Potter & Gergely, 1975).

As a first step toward the understanding of calcium fluxes between the calcium store (sarcoplasmic reticulum), the soluble parvalbumin and the myofibrillar troponin C, Ca^{2+}-free parvalbumins have been shown to bind magnesium (Pechère, 1977; Potter et al., 1977; Cox et al., 1977), the two metals competing for the same sites.

This paper reports the detailed study of Ca^{2+} and Mg^{2+} binding to four different parvalbumins, two from α lineage, rabbit Pa pI = 5.55 and frog Pa pI = 4.88, and two from β lineage, frog Pa pI = 4.50 and hake Pa pI = 4.36. Parvalbumins exhibit two independent Ca^{2+}–Mg^{2+} high-affinity sites, similar to sites III and IV of skeletal troponin C (Potter & Gergely, 1975; Leavis et al., 1978). α- and β-parvalbumins exhibit different affinities for Ca^{2+}, and, among the proteins investigated, one of them (hake pI = 4.36) appeared to exhibit nonequivalent sites and to lose part of its Ca^{2+}-binding capacity upon removal of divalent cations. Besides this first report of differences in ion-binding properties, a general scheme is presented which accounts for the relaxing properties of parvalbumins and provides a rationale for the existence of two classes (low and high affinity) of Ca^{2+}-binding sites in proteins.

Materials and Methods

Materials. All chemicals were reagent grade or the purest commercially available. $^{45}CaCl_2$ (29.7 Ci/g) was obtained from CEA, Gif sur Yvette. Ultrapure water (Milli-Q instrument from Millipore Corp.) and acid-washed plastic ware were used throughout to minimize calcium contamination. Whenever glassware had to be used, it was washed with 50% nitric acid and extensively rinsed with ultrapure water (pCa > 7). Dialysis tubing was boiled in neutral EGTA[1] and rinsed before use. Parvalbumins from rabbit (*Oryctolagus cuniculus*), pI = 5.55, from hake (*Merluccius merluccius*), pI = 4.36, and from frog (*Rana esculenta*), pI = 4.88 and 4.50, were prepared as previously described (Pechère et al., 1971a,b; Capony et al., 1975, 1976).

Methods. The protein concentration of stock parvalbumin solutions was determined by amino acid analysis (Analyzer Beckman Multichrom Model 4255) after 24-h hydrolysis in 6 N HCl at 110 °C by using norleucine as internal standard (Moore & Stein, 1963). Dilutions from stock solutions were checked by ultraviolet spectrophotometry (Beckman Acta III instrument). Calcium and magnesium were measured by atomic absorption spectrophotometry by using a Varian Model 1150 apparatus. Parvalbumins used in this study were found to contain 2 ± 0.2 mol of Ca^{2+}/mol, except for the rabbit protein which, after desalting in the presence of 0.2 M acetic acid, contained only 0.15 mol of Ca^{2+}/mol.

Equilibrium-dialysis experiments were carried out on parvalbumins freed from Ca^{2+}(<0.1 mol/mol) by dialysis vs. 50 vol of 0.15 M KCl, 10 mM EGTA, 25 mM Hepes buffer, pH 7.55, for at least 3 days at +4 °C. Magnesium binding was studied on 0.1–0.3 mM parvalbumin, dialyzed 3 days at +4 °C vs. eight changes of 50 vol of 0.15 M KCl, 25 mM Hepes calcium-free buffer, pH 7.55, containing variable amounts of magnesium acetate. Protein and metal concentrations were then determined both within the dialysis bag and in the outer medium.

Flow-dialysis experiments were performed at room temperature (22 ± 3 °C) according to Colowick & Womack (1969). Parvalbumins were freed from Ca^{2+} as described above and then dialyzed vs. 0.15 M KCl, 25 mM Hepes buffer, pH 7.55, passed through Chelex 100 (200–400 mesh, from Bio-Rad). The upper compartment of the flow dialysis cell contained 4 mL of 0.1 mM parvalbumin, 1–1.5 μM $^{45}CaCl_2$, 0.15 M KCl, 25 mM Hepes buffer, pH 7.55. The lower compartment was perfused with the Hepes–KCl buffer (240 mL/h), collecting 1.2-mL fractions. Ca^{2+} (100 nmol) was added every 3 min, until the final chase (20 μmol). From each fraction, 1 mL was withdrawn and counted for 5 min in 10 mL of dioxane–naphthalene scintillant. Competition by magnesium was studied by adjusting both upper and lower compartment buffer to the adequate Mg^{2+} concentration.

Differential UV spectrophotometry was carried out on Varian instruments Cary 118 and Cary 219. Titration of Ca^{2+}-free parvalbumins by Mg^{2+} was performed by sequential additions of magnesium acetate (up to 5 mM) to 0.3 or 0.6 mM parvalbumin, 0.15 M KCl, 25 mM Mops buffer, pH 7.2. To minimize interference from base-line drift, the maximal absorbance difference between a peak and a trough was measured and plotted vs. the molar ratio Mg^{2+}/parvalbumin.

Similarly, Ca^{2+}-loaded parvalbumins were titrated by addition of EGTA (up to 10 mM unless otherwise specified) in 0.15 M KCl, 100 mM Hepes buffer, pH 7.55. The absorbance difference between the 264.5-nm trough and the 267-nm peak was used for calculations.

The relative error $\Delta(\Delta\epsilon)/\Delta\epsilon$ is the sum of relative errors on parvalbumin concentration (3% by amino acid analysis) and on ΔA. Absorbance measurements were performed with a precision of 5×10^{-4} absorbance units, and $\Delta(\Delta A)/\Delta A$ ranged from 1 to 10%. Total relative error therefore ranged from 4 to 13%.

In both types of experiments, buffer alone was added to the reference half-cell. At the end of each experiment, the absence of protein precipitation was checked.

The determination of the number of Ca^{2+}-binding sites present in the native, Ca^{2+}-loaded, hake parvalbumin was carried out on 0.223 mM parvalbumin in 0.15 M KCl, 25 mM Hepes buffer, pH 7.55. Total Ca^{2+} was measured by atomic absorption spectrophotometry. Bound Ca^{2+} was determined by a flow-dialysis experiment, performed as described above on the protein solution (1 mL) containing 16.8 nmol of $^{45}CaCl_2$, from the radioactivity difference between the plateaus obtained before and after addition of 10 μmol of $CaCl_2$.

Analysis of Binding Data. In our experiments of equilibrium dialysis and flow dialysis, data were plotted according to Scatchard (1949), and linear regression by the least-squares method was carried out on an Olivetti P 6060 minicomputer.

Analysis of Data Obtained by Differential UV Spectrophotometry. The binding of Ca^{2+} to Pa may be represented as

$$\mathrm{Pa} + \mathrm{Ca} \underset{}{\overset{K_1}{\rightleftharpoons}} \mathrm{PaCa} + \mathrm{Ca} \overset{K_2}{\rightleftharpoons} \mathrm{PaCa_2}$$

where

$$K_1 = \frac{[\mathrm{PaCa}]}{[\mathrm{Pa}]x} \quad (1)$$

$$K_2 = \frac{[\mathrm{PaCa_2}]}{[\mathrm{PaCa}]x} \quad (2)$$

[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; UV, ultraviolet; Pa, parvalbumin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; SR, sarcoplasmic reticulum; ν, number of metal-binding sites/mole of protein; Tris, tris(hydroxymethyl)-aminomethane; Pipes, 1,4-piperazinediethanesulfonic acid.

and x = free Ca^{2+} concentration.

Titration of Ca^{2+}-loaded Pa with EGTA affects the UV-absorbing properties of the protein. The molar extinction coefficients of Pa, PaCa, and $PaCa_2$ are ϵ_0, ϵ_1, and ϵ_2, respectively, and $\Delta\epsilon$ is the variation in the molar extinction coefficient of Pa after EGTA addition.

Therefore

$$\Delta\epsilon^{E_t} = \epsilon_0[\alpha_0^{E_t} - \alpha_0^0] + \epsilon_1[\alpha_1^{E_t} - \alpha_1^0] + \epsilon_2[\alpha_2^{E_t} - \alpha_2^0]$$

where E_T denotes the total concentration of EGTA, i.e.

$$E_T = [\text{EGTA·Ca}] + [\text{EGTA}] \quad (3)$$

and α_i denotes the mole fraction of Pa binding i moles of Ca^{2+} in the presence (superscript E_T) or absence (superscript 0) of EGTA (Gill et al., 1978).

$$\Delta\epsilon^{E_t} = [\epsilon_0\alpha_0^{E_t} + \epsilon_1\alpha_1^{E_t} + \epsilon_2(1 - \alpha_1^{E_t} - \alpha_0^{E_t})] - [\epsilon_0\alpha_0^0 + \epsilon_1\alpha_1^0 + \epsilon_2(1 - \alpha_1^0 - \alpha_0^0)]$$

since

$$\alpha_1 + \alpha_2 + \alpha_0 = 1 \quad (4)$$

i.e. $\alpha_2 = 1 - \alpha_1 - \alpha_0$. Therefore

$$\Delta\epsilon^{E_t} = \alpha_0^{E_T}\left[(\epsilon_0 - \epsilon_2) + \frac{\alpha_1^{E_T}}{\alpha_0^{E_T}}(\epsilon_1 - \epsilon_2)\right] - \alpha_0^0\left[(\epsilon_0 - \epsilon_2) + \frac{\alpha_1^0}{\alpha_0^0}(\epsilon_1 - \epsilon_2)\right] \quad (5)$$

From eq 1, $K_1 = \alpha_1/(\alpha_0 x)$ or $\alpha_1/\alpha_0 = K_1 x$. From eq 1 and 2, $K_1K_2 = \alpha_2/(\alpha_0 x^2)$, and from eq 4, $\alpha_0 = 1/(1 + K_1x + K_1K_2x^2)$. Therefore

$$\Delta\epsilon^{E_t} = \frac{(\epsilon_0 - \epsilon_2) + (\epsilon_1 - \epsilon_2)K_1x}{1 + K_1x + K_1K_2x^2} - \frac{(\epsilon_0 - \epsilon_2) + (\epsilon_1 - \epsilon_2)K_1x_0}{1 + K_1x_0 + K_1K_2x_0^2} = \frac{A_1 + A_2K_1x}{1 + K_1x + K_1K_2x^2} - \frac{A_1 + A_2K_1x_0}{1 + K_1x_0 + K_1K_2x_0^2} \quad (6)$$

in which $A_1 = \epsilon_0 - \epsilon_2$, $A_2 = \epsilon_1 - \epsilon_2$, and x_0 is the initial free calcium concentration before EGTA addition. The system is defined by eq 1–3 and by:

$$K = \frac{[\text{EGTACa}]}{[\text{EGTA}]x} \quad (7)$$

$$Pa_T = [Pa] + [PaCa] + [PaCa_2] \quad (8)$$

$$Ca_T = x + [PaCa] + 2[PaCa_2] + [\text{EGTACa}] = x + \frac{(K_1Pa_Tx) + 2Pa_TK_1K_2x^2}{1 + K_1x + K_1K_2x^2} + \frac{KE_Tx}{1 + Kx} \quad (9)$$

The value of x is obtained from the equation:

$$x = \frac{Ca_T}{1 + \dfrac{Pa_TK_1 + 2K_1K_2Pa_Tx}{1 + K_1x + K_1K_2x^2} + \dfrac{KE_T}{1 + Kx}} \quad (10)$$

x is then used in eq 6 to obtain a set of values for K_1, K_2, A_1, and A_2. The fitting of the theoretical curve to the experimental points was performed on an Olivetti P 6060 minicomputer, in order to get the minimal value of χ^2, defined by:

$$\chi^2 = \frac{1}{N}\sum_{i=1}^{N}\left(\frac{\Delta\epsilon_{exp} - \Delta\epsilon_{calcd}}{\Delta\epsilon_{exp}}\right)$$

where N is the number of experimental points.

Table I: Magnesium and Calcium Binding to Parvalbumins Studied by Equilibrium and Flow Dialysis

		rabbit Pa pI = 5.55	frog Pa pI = 4.88	frog Pa pI = 4.50	hake Pa pI = 4.36
		Mg^{2+} Binding			
	K_d (μM)	16	21	27	30
	ν	2.3	1.9	1.9	1.4[a]
		Ca^{2+} Binding in the Presence of Mg^{2+}			
no Mg^{2+}	ν	ND[c]	ND	ND	1.0 ± 0.2
11.25 mM	K_d (μM)				1.5
	ν				1.1
12.5 mM	K_d (μM)				1.8
	ν				1.1
22.5 mM	K_d (μM)				2.2
	ν				1.3
50 mM	K_d (μM)	15	11	6	6.1[b]
	ν	2.0	1.7	1.5	1.5
100 mM	K_d (μM)	38	31	10	17
	ν	1.7	1.9	2.0	1.3
112.5 mM	K_d (μM)	ND	ND	ND	9
	ν				1.1
150 mM	K_d (μM)	55	48	14	16
	ν	2.0	1.3	2.0	1.3[b]
calcd	$K_{dCa^{2+}}$ (nM)[d]	6.6	7.8	2.2	3.2

[a] Determination of the number of sites at saturating Mg^{2+} pointed to $\nu = 1.0 \pm 0.34$ ($\bar{X} \pm$ SD, $n = 4$). [b] Experiments performed in the presence of 1 mM DTT indicated K_d values of 4.0 μM (1.4 sites) and 9.7 μM (1.5 sites) in the presence of 50 and 150 mM Mg^{2+}, respectively. [c] ND = not determined. [d] $K_{dCa^{2+}}$ was computed as described under Results.

From the values of K_1 and K_2, the solutions $x' = -1/K_{d_1}$ and $x'' = -1/K_{d_2}$ of the binding polynomial $1 + K_1x + K_1K_2x^2 = 0$ can be calculated (Wyman, 1965).

Results

Magnesium Binding to Parvalbumins. Equilibrium dialysis of Ca^{2+}-free parvalbumin vs. Mg^{2+} containing buffer shows unequivocally that parvalbumin is capable of binding Mg^{2+} with a K_d from 16 to 30 μM (Table I). There are, however, differences between parvalbumins. Mg^{2+} binding to α-parvalbumins is somewhat tighter (K_d = 16 and 21 μM) than Mg^{2+} binding to the β-proteins (27 and 30 μM). The main difference lies, however, in the number of sites obtained from the X-axis intercept of Scatchard plots.[2] Two independent and equivalent sites are clearly present in the rabbit protein and in both frog components. On the contrary, a similar experiment carried out with hake parvalbumin indicated 1.4 sites when a single line was drawn ($r^2 = 0.97$). Alternatively, binding data could be interpreted as indicative of two non-equivalent sites (K_{d_1} = 17 μM and K_{d_2} = 0.4 mM).[2] However, another experiment performed at higher protein (0.5–1 mM) and magnesium (1–5 mM) concentrations, pointed to $\nu = 1.0 \pm 0.34$ ($\bar{X} \pm$ SD, $n = 4$).

Direct titration of Ca^{2+}-free parvalbumins by Mg^{2+} was performed by differential UV spectroscopy, which takes advantage of the conformational changes induced by ion binding. Such changes are accompanied by shifts in the absorbance of the aromatic rings packed into the hydrophobic core of the molecule (Moews & Kretsinger, 1975a).

Mg^{2+} uptake by ion-free parvalbumins can thus be easily followed between 240 and 310 nm, as shown in Figure 1. α-Proteins, i.e., rabbit pI = 5.55 and frog pI = 4.88, exhibit

[2] Not shown, but submitted to reviewers for examination. The material will be sent upon request to interested readers.

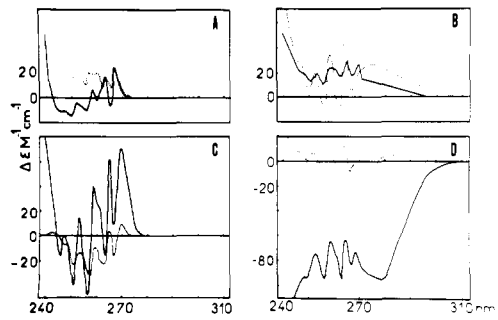

FIGURE 1: Differential spectra of parvalbumins. (—) Magnesium substituted (10 mM Mg^{2+}) vs. metal free; (···) calcium substituted (10 mM Ca^{2+}, 10 mM Mg^{2+}) vs. magnesium substituted (10 mM Mg^{2+}). (A) Rabbit pI = 4.55; (B) frog pI = 4.88; (C) hake pI = 4.36; (D) frog pI = 4.50.

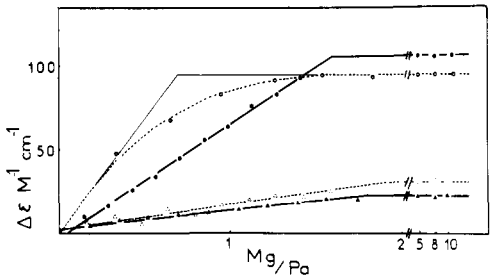

FIGURE 2: Titration of ion-free parvalbumins by Mg^{2+} ions. Conformational changes induced by successive additions of Mg^{2+} to parvalbumins were followed by differential UV spectroscopy as described under Methods, and the molar maximal absorbance difference was plotted vs. the molar ratio Mg^{2+}/pavalbumin. For hake parvalbumin, the initial slope was extrapolated to the plateau level (end point: 0.65 mol of Mg^{2+}/mol of Pa). (O---O) Hake pI = 4.36; (●—●) frog pI = 4.50; (Δ---Δ) frog pI 4.88; (▲—▲) rabbit pI = 5.55.

a weaker difference signal than the β-proteins, hake pI = 4.36 and frog pI = 4.50. The latter component shows, on top of the vibronic structure due to the core phenylalanyl side chains, a marked hypochromicity of the single tyrosyl residue located in position 26 (Capony et al., 1975) of the peptide chain. This position is occupied by a histidine residue in the other three parvalbumins.

The amplitude of the difference signal ($\Delta\epsilon$) plateaus when the high-affinity sites are saturated by magnesium, as shown in Figure 2. For α-parvalbumins from rabbit and frog (pI = 4.88) which exhibited a negligible amount of residual bound Ca^{2+} (<0.1 mol/mol), the plateau was reached at 1.8 and 1.9 mol of Mg^{2+}/mol, confirming the existence of two independent and equivalent binding sites, which cannot be distinguished on the basis of the ion-induced conformational changes. Similar conclusions can be drawn from the titration of frog pI = 4.50 by 1.6 mol of Mg^{2+}/mol since this particular protein sample was found to be still contaminated by 0.3 mol of Ca^{2+}/mol.

By contrast, the titration curve of hake parvalbumin is nonlinear; extrapolations of the initial increase in $\Delta\epsilon$ and of the plateau intersect at 0.65 mol of Mg^{2+}/mol. This sample was found to contain 0.1–0.2 mol of residual bound Ca^{2+}/mol.

The results presented above are consistent with the presence in Ca^{2+}-free parvalbumins of two high-affinity Mg^{2+}-binding sites, except for the hake protein.

Competition of Magnesium and Calcium for the Same Sites. Flow-dialysis experiments performed in the presence of ^{45}Ca and of various Mg^{2+} concentrations (see Table I) show that addition of Mg^{2+} does not change the number of Ca^{2+}-binding sites but decreases equally their affinity for Ca^{2+}, obtained from Scatchard plots.[2] These results are in agreement with a simple competition of both metals for the same sites. The apparent dissociation constant for calcium obeys the relation

$$K_{dCa^{2+}app} = K_{dCa^{2+}}\left(1 + \frac{[Mg^{2+}]}{K_{dMg^{2+}}}\right)$$

When $K_{dCa^{2+}app}$ is plotted vs. $[Mg^{2+}]$, the intercept $K_{dCa^{2+}}$ is too small to be determined with accuracy, whereas the slope $K_{dCa^{2+}}/K_{dMg^{2+}}$ can be obtained by the least-squares method. From the slope, $[Mg^{2+}]$, and $K_{dCa^{2+}app}$, $K_{dCa^{2+}}$ was calculated (see Table I).

The affinity for Ca^{2+} appears to be somewhat weaker for α ($K_{dCa^{2+}}$ = 6.6 and 7.8 nM) than for β-parvalbumins (2.2 and 3.2 nM).

Both frog and the rabbit parvalbumins exhibit two independent and equivalent calcium-binding sites throughout the Mg^{2+} concentration range. Hake parvalbumin also shows simple competition of both metals for the same sites. The experimental values of ν lie between 1.0 and 1.45, but only a single straight line could be drawn through the experimental points (r^2 = 0.99).

Structural transitions occurring when Ca^{2+} ions displace Mg^{2+} from the binding sites were studied by differential UV spectroscopy (see Figure 1). In the four parvalbumins, addition of Ca^{2+} to Mg^{2+}-containing parvalbumin induces structural changes and alters the peak pattern of the aromatic residues of the hydrophobic core. This structural change is of physiological importance since it is occurring in the cytosol when Ca^{2+} ions are released from the terminal cisternae. Whereas absorption changes induced by ion exchange in α-parvalbumins are of the same order of magnitude as those induced by Mg^{2+} addition to the metal-free proteins, β-parvalbumins exhibit much smaller changes upon ion exchange than upon ion binding. They are therefore likely to possess the same overall structure whether they bind Ca^{2+} or Mg^{2+}. Similar findings were previously reported for troponin C (Levine et al., 1978). The contrast between the ion exchange on the one hand and addition of Mg^{2+} to the metal-free protein on the other hand is evident for frog pI = 4.50 parvalbumin, in which only a weak hyperchromicity of Tyr-26 is associated with the exchange (see Figure 1D).

Calcium-Binding Properties of Native Hake Parvalbumin. The particular behavior of hake parvalbumin, which, in its Ca^{2+}-free form, appears to exhibit less than two Ca^{2+}–Mg^{2+} sites, prompted the experiment, described under Methods, to measure accurately the number of bound Ca^{2+} in the native Ca^{2+}-loaded protein, which contained 1.9 mol of Ca^{2+}/mol.

The molecule could have lost part of its Ca^{2+}-binding capacity during the prolonged dialysis for calcium removal. Ca^{2+} binding was therefore studied the other way around, starting from the Ca^{2+}-substituted protein and following by differential UV spectroscopy the structural changes occurring when Ca^{2+} is removed through additions of EGTA (Figure 3). Experiments were carried out at several pH values from 6.8 to 8.0 and, for each experiment, the experimental points were automatically fitted (see Methods) with a theoretical curve. The set of parameters K_1, K_2, A_1, A_2 and the calculated dissociation constants K_{d1} and K_{d2} are shown in Table II.

The data are best interpreted in terms of two sites. The high-affinity site K_d value (3–5 nM) is in excellent agreement with the value obtained from flow-dialysis experiments for the high-affinity site present in Ca^{2+}-free parvalbumin. The second binding site shows an affinity ca. 1 order of magnitude lower, with K_d ranging from 17 to 60 nM, its determination being obviously less accurate.

Table II: Hake Parvalbumin Dissociation Constants for Ca^{2+} Obtained by UV Differential Spectroscopy

conditions	K_{app} EGTA $\times 10^{-5}$ at exptl pH[a] (M^{-1})	$K_1 \times 10^{-8}$ (M^{-1})	$K_2 \times 10^{-7}$ (M^{-1})	A_1 (M^{-1} cm^{-1})	A_2 (M^{-1} cm^{-1})	$\chi^2 \times 10^3$	K_{d_1} (nM)	K_{d_2} (nM)
0.1M Hepes, 0.15M KCl, pH 7.55 $Pa_T = 291\ \mu M$	163	2.8	4.0	257	26	10	4	21
0.1M Hepes, 0.15M KCl, pH 7.55 $Pa_T = 355\ \mu M$	163	4.0	5.0	258	17	2	3	17
0.1M Hepes, 0.15M KCl, pH 7.65 $Pa_T = 152\ \mu M$	254	2.5	4.0	261	16	1	5	20
0.1M Pipes, 0.15M KCl, pH 6.8 $Pa_T = 339\ \mu M$	5.37	3.2	1.6	253	44	5	4	60
0.1M Tris, pH 8.0 $Pa_T = 390\ \mu M$	4552	4.0	5.0	264	92	2	3	17

[a] K_{app} values for EGTA were computed from Godt (1974) except for the value at pH 8.0, computed from Schwarzenbach et al. (1975), as explained under Results.

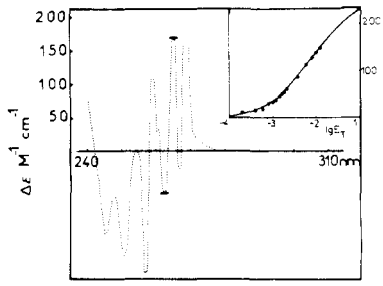

FIGURE 3: Differential spectrum of hake parvalbumin pI = 4.36 (0.291 mM) containing 2.2 Ca^{2+}/mol vs. the protein freed from calcium by addition of EGTA at pH 7.55 (see Methods). Signal between bars was measured and plotted vs. log [EGTA] (lg E_T) and a theoretical curve was fitted to the experimental points (inset).

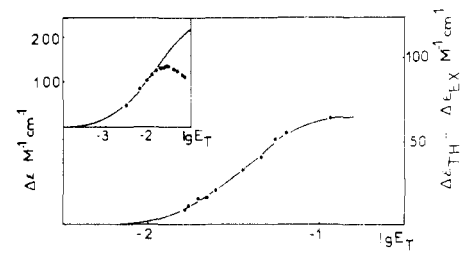

FIGURE 4: Binding isotherm of EGTA to hake parvalbumin. Hake Pa pI = 4.36 (0.355 mM) was titrated by EGTA at pH 7.55 (see Figure 3), and a theoretical curve was fitted to the experimental points at low EGTA concentration. At higher EGTA concentrations, the difference between the theoretical curve, which assumes no protein–EGTA interaction, and the experimental points increases with EGTA concentration (inset). This difference ($\Delta\epsilon_{TH} - \Delta\epsilon_{EX}$) was plotted vs. log [EGTA] (lg E_T). Half-maximal difference, i.e., half-maximal binding ($K_{0.5}$), was observed at 35 mM EGTA.

In this respect, binding of Ca^{2+} at the two sites seems unaffected by pH between 6.8 and 7.65, where EGTA affinity constants for Ca^{2+} were taken from Godt (1974). Had the same source been chosen for the EGTA constant at pH 8.0, the K_1 value would have been 5.0×10^7, meaning that affinity of parvalbumin for Ca^{2+} would change abruptly between pH 7.65 and 8.0. As this seems unlikely, K_{EGTA} at this pH was taken from Schwarzenbach et al. (1957), giving K_d values for parvalbumin in good agreement with figures obtained at lower pH.

The experimental approach used above also provides further information about the binding of EGTA to parvalbumin. EGTA binding to the protein is suggested by the signal amplitude decrease when EGTA concentration is increased above 10 mM at pH 7.55 in the presence of 0.35 mM protein. When the protein concentration is lower, the concentration of EGTA which induces a signal decrease is higher, pointing to a protein restructuration induced by EGTA binding. An approximate apparent dissociation constant of 35 mM was obtained from the differences between the theoretical curve, which assumes no EGTA–protein interaction, and the experimental points (Figure 4).

Discussion

The present report provides evidence for the existence of two high-affinity Ca^{2+}–Mg^{2+} sites in parvalbumins, similar to those of domains III and IV of troponin C (Potter & Gergely, 1975; Leavis et al., 1978). These data confirm previous reports on Mg^{2+}–Ca^{2+} competition in parvalbumins (Pechère, 1977; Potter et al., 1977; Cox et al., 1977). However, they are not in accord with the hypothesis (Potter et al., 1977) according to which Ca^{2+}–Mg^{2+} sites do not contain a glycyl residue between the Y and Z coordination sites, in contrast to the Ca^{2+}-specific sites. Among the ten parvalbumin binding sites (this study, and carp 4.25 (Potter et al., 1977)) which have been shown to be high-affinity Ca^{2+}–Mg^{2+} sites, eight amino acid sequences are known, since the primary structure of frog pI = 4.88 has not yet been determined. All CD sites (Moews & Kretsinger, 1975a) exhibit a lysyl residue in this position, while all EF sites exhibit a glycyl residue (Table III). Assuming that our sample on two α- and two β-parvalbumins is representative of parvalbumins in general, the 14 amino acid sequences known at the moment (see Kretsinger, 1979, for review) all show this glycine in the EF site between the Y and Z coordination sites, while it is never present in the corresponding position of the CD site, occupied mostly by lysine, and in one case each by alanine and by glutamine.

The peculiar behavior of hake parvalbumin pI = 4.36, which appears to lose up to one site upon removal of Ca^{2+} ions, may perhaps be explained in different ways.

Either one of the two sites is somewhat more labile than in the other parvalbumins, and, in this respect, the presence in the CD loop of aspartyl and valyl residues between the Z and –Y, and –Y and –X coordination sites, respectively, is remarkable, since all other parvalbumins show invariant glycine and isoleucine in these positions (see Table III). This hypothesis does not, however, account for the nonintegral values (1.0–1.5) obtained for the number of sites in the Ca^{2+}-free protein. Another explanation may be the formation of a

Table III: Amino Acid Sequences of Ca^{2+}-Mg^{2+}-Binding Sites in Parvalbumins[a]

parvalbumin	site	X		Y		Z		−Y		−X			−Z
rabbit pI = 5.55[b]	CD	D	K	D	K	S	G	F	I	E	E	E	E
	EF	D	K	D	G	D	G	K	I	G	A	D	E
frog pI = 4.50[b]	CD	D	R	D	K	S	G	F	I	E	Q	D	E
	EF	D	S	D	G	D	G	K	I	G	V	E	E
hake pI = 4.36[b]	CD	D	Q	D	K	S	D	F	V	E	E	D	E
	EF	D	S	D	G	D	G	K	I	G	V	E	E
carp pI = 4.25[c]	CD	D	Q	D	K	S	G	F	I	E	E	D	E
	EF	D	S	D	G	D	G	K	I	G	V	D	E

[a] The coordinating ligands are designated according to Moews & Kretsinger (1975a). [b] Taken from Goodman & Pechère (1977). [c] Shown to exhibit two Ca^{2+}-Mg^{2+}-binding sites by Potter et al. (1977).

disulfide bond between the single cysteinyl side chains of the protein. There are indeed indications, from NMR experiments (B. Levine and J. Haiech, unpublished data), that some dimerization occurs in ion-free parvalbumin solutions. However, flow-dialysis experiments and spectrophotometric titrations by Mg^{2+} performed in the presence of 1 mM dithiothreitol did show the same number of sites, with perhaps only a slight increase in the affinity for Ca^{2+} (see Table I). Binding data obtained from equilibrium dialysis of the Ca^{2+}-free protein in the presence of Mg^{2+} may also be interpreted as indicative of two nonequivalent sites, with K_{d2}/K_{d1} = ca. 23. This ratio is much larger than the one observed with affinities for Ca^{2+}, as measured by UV differential spectrophotometry. Moreover, starting from the metal-free protein, the two binding sites could never be saturated by Ca^{2+} or Mg^{2+}, even at concentrations more than ten times higher than the dissociation constant of the lower affinity site. Therefore, the hypothesis of an homogeneous protein population, showing two nonequivalent sites, is also not satisfactory.

It is also obvious that titration by EGTA of a Ca^{2+}-loaded protein, followed by UV differential spectrophotometry, is more sensitive in distinguishing two sites with affinities for Ca^{2+} differing by ca. one order of magnitude than is the flow-dialysis technique. There are other indications that the sites may not be strictly equivalent. One of the ligands of the EF sites has been shown to be a water molecule, by contrast with the CD site, where all the coordination sites are contributed by the polypeptide chain (Moews & Kretsinger, 1975a). The binding of lanthanides and other metals is also different for the two sites. For instance, Tb(III) occupies the EF site first (Moews & Kretsinger, 1975b; Sowadski et al., 1978). Also ^{113}Cd NMR spectroscopy points to nonequivalence of CD and EF sites, especially with respect to Gd(III) ion binding (Drakenberg et al., 1978).

The presence or absence of cooperativity between the two sites is still unclear. From the tertiary structure of parvalbumin, a model has been proposed in which Ca^{2+} bound to the EF site would dissociate first, facilitating the removal of the CD Ca^{2+} (Kretsinger & Nockolds, 1973). The methods used in this study would not distinguish such a cooperativity from a model with two independent sites of different affinity. Other reports (Nelson et al., 1976) do not favor a cooperativity between the two sites. Our results show no evidence for such a cooperativity. In three proteins from frog and rabbit, the sites can be considered as independent and equivalent high-affinity Ca^{2+}-Mg^{2+}-binding sites. Hake parvalbumin can be treated as well as presenting two independent sites of different affinities for Ca^{2+}.

This report also presents the first evidence for differences in Ca^{2+}-binding properties between proteins of the two genetic lineages α and β. β-Parvalbumins appear to bind Ca^{2+} more strongly than the α-proteins. The somewhat intermediate value of $K_{dCa^{2+}}$ exhibited by hake parvalbumin is perhaps due to the aspartyl residue present between the Z and −Y coordination sites or to partial dimerization of the protein, involving the cysteine-20 present in the AB loop, that may influence Ca^{2+}-binding properties, as discussed below. The other parvalbumins do not show significant differences in the structure of their binding sites, and the overall tertiary structure is likely to account for the differences in affinity for Ca^{2+} ions in the two genetic lines. It has been postulated, indeed, that different protein conformations will result in different affinities for Ca^{2+} (Kretsinger, 1977).

The existence of high-affinity Ca^{2+}-Mg^{2+} binding sites in parvalbumins provides a coherent explanation for their physiological role. In resting muscle (pCa is approximately 8), the magnesium concentration is 1–6 mM (Brinley et al., 1977), and parvalbumins are present as $PaMg_2$, since the $K_{dMg^{2+}}$ is in the range of $2–3 \times 10^{-5}$ M for both sites. When Ca^{2+} is liberated from SR vesicles at the onset of contraction, Mg^{2+} ions are displaced by Ca^{2+}. Therefore, the conformational changes occurring upon binding of Ca^{2+} to the ion-free protein, although extensively studied (Parello & Pechère, 1971; Parello et al., 1974; Opella et al., 1974; Donato & Martin, 1974), have no physiological significance, in contrast to those occurring upon Ca^{2+}-Mg^{2+} exchange. These changes are much smaller, but can still be followed by UV spectroscopy. A major finding is that Ca^{2+}-Mg^{2+} exchange induces structural changes in the entire molecule, as shown by the absorption change of the single tyrosyl residue in position 26 of the linear sequence of frog pI = 4.50 protein. This residue belongs to the AB loop, opposite to the CD and EF sites and close to the invariant salt bridge between arginine-75 and glutamic acid-81, which could play an important role in the calcium-binding properties of parvalbumin (Gosselin-Rey et al., 1973).

The Mg^{2+}-binding properties of parvalbumin also explain why parvalbumin, present in high concentration between the SR terminal cisternae and myofibrils, do not prevent Ca^{2+} ions from reaching the Ca^{2+}-specific sites of troponin C, which trigger contraction (Potter & Gergely, 1975). The dissociation of Mg^{2+} ions occurs indeed with a $t_{1/2}$ = ca. 23–230 ms, for a $K_{dMg^{2+}} = 3 \times 10^{-5}$ M and a binding rate constant $k_1 = 10^6$ to 10^7 M^{-1} s^{-1} corresponding to diffusion-limited binding kinetics. The delay in Ca^{2+}-binding caused by Mg^{2+} dissociation allows Ca^{2+} binding to the Ca^{2+}-specific sites of troponin C or calmodulin (calcium-dependent regulator) (Potter et al., 1977) that are not occupied by Mg^{2+}. Later on, however, the high affinity Ca^{2+}-Mg^{2+} sites should be able to compete successfully for calcium and help remove Ca^{2+} from the Ca^{2+}-specific sites of troponin C and calmodulin, inducing relaxation, e.g.

Parvalbumin-bound Ca^{2+} is then removed by the SR pump (Figure 5). Such a scheme is in perfect agreement with the hypothesis describing parvalbumin as a soluble relaxing factor and with the experimental results obtained in this connection

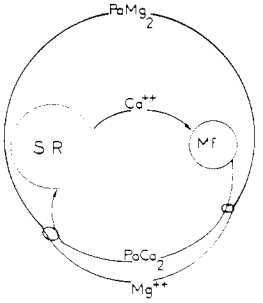

FIGURE 5: Scheme of the calcium cycle in fast muscle fibers. Mf, myofibril; SR, sarcoplasmic reticulum. Parvalbumin binds 2 Mg^{2+} ions in resting muscle (pCa 8). Ca^{2+} released from SR binds first to the Ca^{2+}-specific sites of troponin C since they are not occupied by Mg^{2+} (contraction). As soon as Mg^{2+} dissociates from parvalbumin, parvalbumin removes Ca^{2+} from troponin C (relaxation), and, in turn, SR removes calcium from parvalbumin, which then binds 2 Mg^{2+} ions.

(Pechère, 1977; Gerday & Gillis, 1976; Pechère et al., 1977).

The data obtained on parvalbumin can be generalized to the other Ca^{2+}–Mg^{2+} sites described in troponin C from skeletal (Potter & Gergely, 1975) and cardiac muscle (Potter et al., 1977). All these sites exhibit $pK_{dCa^{2+}}$ above 7 and $pK_{dMg^{2+}}$ above 3, with differences $pK_{dCa^{2+}} - pK_{dMg^{2+}}$ of ca. 4. They can be considered as "relaxing sites".

By contrast, Ca^{2+}-specific sites exhibit $pK_{dCa^{2+}}$ values below 6. They are likely to bind magnesium with a $pK_d < 3$. This binding would be difficult to demonstrate and would have in any case no physiological significance. Such sites, present in troponin C or calmodulin, known to trigger Ca^{2+}-mediated events, are best described as "triggering sites". There are preliminary indications that Ca^{2+}–Mg^{2+} sites evolved from Ca^{2+}-binding sites in the course of the evolution of Ca^{2+}-binding proteins (M. Goodman, J. F. Pechère, J. Haiech, and J. G. Demaille, unpublished). Such a Darwinian evolution allowed the use of the same basic EF hand structure (Kretsinger, 1977) to fulfill two different functions, the triggering by Ca^{2+} of a metabolic process, and its rapid termination upon Ca^{2+} removal. This accounts also for the presence of parvalbumin in nervous tissue (Baron et al., 1975), in which rapid Ca^{2+} fluxes are associated with synaptic transmission.

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References

Baron, G., Demaille, J., & Dutruge, E. (1975) *FEBS Lett. 56*, 156–160.

Benzonana, G., Capony, J.-P., & Pechère, J.-F. (1972) *Biochim. Biophys. Acta 278*, 110–116.

Blaustein, M. P., Ratzlaff, R. W., Kendrick, N. C., & Schweitzer, E. S. (1978) *J. Gen. Physiol. 72*, 15–41.

Blum, H. E., Lehky, P., Kohler, L., Stein, E., & Fischer, E. H. (1977) *J. Biol. Chem. 252*, 2834–2838.

Brinley, F. J., Scarpa, A., & Tiffert, T. (1977) *J. Physiol. 266*, 545–565.

Capony, J.-P., Demaille, J., & Pechère, J.-F. (1975) *Eur. J. Biochem. 56*, 215–227.

Capony, J.-P., Pina, C., & Pechère, J.-F. (1976) *Eur. J. Biochem. 70*, 123–135.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.

Cox, J. A., Wnuk, W., & Stein, E. A. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) pp 266–269, North Holland, New York.

Demaille, J., Dutruge, E., Eisenberg, E., Capony, J.-P., & Pechère, J.-F. (1974) *FEBS Lett. 42*, 173–178.

Donato, H., & Martin, R. B. (1974) *Biochemistry 13*, 4575–4579.

Drakenberg, T., Lindman, B., Cavé, A., & Parello, J. (1978) *FEBS Lett. 92*, 346–350.

Gerday, C., & Gillis, J. M. (1976) *J. Physiol. 258*, 96–97P.

Gill, S. J., Gaud, H. T., Wyman, J., & Barisas, B. J. (1978) *Biophys. Chem. 8*, 53–59.

Godt, R. E. (1974) *J. Gen. Physiol. 63*, 722–739.

Goodman, M., & Pechère, J.-F. (1977) *J. Mol. Evol. 9*, 131–158.

Gosselin-Rey, C., Bernard, N., & Gerday, C. (1973) *Biochim. Biophys. Acta 303*, 90–104.

Hasselbach, W. (1978) *Biochim. Biophys. Acta 515*, 23–53.

Kretsinger, R. H. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., et al., eds.) pp 63–72, North Holland, New York.

Kretsinger, R. H. (1979) *Crit. Rev. Biochem. (in press)*.

Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248*, 3313–3326.

Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452–5459.

Levine, B. A., Thornton, J. M., Fernandes, R., Kelly, C. M., & Mercola, D. (1978) *Biochim. Biophys. Acta 535*, 11–24.

Moews, P. C., & Kretsinger, R. H. (1975a) *J. Mol. Biol. 91*, 201–228.

Moews, P. C., & Kretsinger, R. H. (1975b) *J. Mol. Biol. 91*, 229–232.

Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–831.

Nelson, D. J., Opella, S. J., & Jardetzky, O. (1976) *Biochemistry 15*, 5552–5560.

Opella, S. J., Nelson, D. J., & Jardetzky, O. (1974) *J. Am. Chem. Soc. 96*, 7157–7159.

Parello, J., & Pechère, J.-F. (1971) *Biochimie 53*, 1079–1083.

Parello, J., Cavé, A., Puigdomenech, P., Maury, C., Capony, J.-P., & Pechère, J.-F. (1974) *Biochimie 56*, 61–76.

Pechère, J.-F. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) pp 213–221, North Holland, New York.

Pechère, J.-F., Capony, J.-P., & Rydén, L. (1971a) *Eur. J. Biochem. 23*, 421–428.

Pechère, J.-F., Demaille, J., & Capony, J.-P. (1971b) *Biochim. Biophys. Acta 236*, 391–408.

Pechère, J.-F., Capony, J.-P., & Demaille, J. (1973) *Syst. Zool. 22*, 533–548.

Pechère, J.-F., Derancourt, J., & Haiech, J. (1977) *FEBS Lett. 75*, 111–114.

Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633.

Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L., & Means, A. R. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) pp 239–250, North Holland, New York.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.

Schwarzenbach, G., Senn, H., & Anderegg, G. (1957) *Helv. Chim. Acta 40*, 1886–1900.

Sowadski, J., Cornick, G., & Kretsinger, R. H. (1978) *J. Mol. Biol. 124*, 123–132.

Wyman, J. (1965) *J. Mol. Biol. 11*, 631–644.