Kinetic Mechanism of Calcium Binding to Whiting Parvalbumin†

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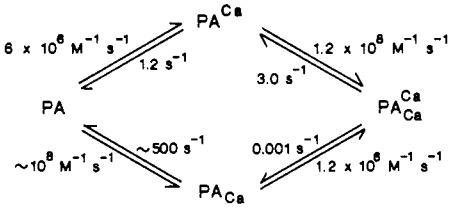
ABSTRACT: Calcium binding to whiting parvalbumin induces large changes in the fluorescence, absorption, and circular dichroism spectra of the protein. The fluorescence emission maximum of the single tryptophan shifts from 325 to 348 nm upon the removal of calcium and decreases in intensity by 50%. All of the spectral changes are linear between 0 and 2 mol of calcium bound/mol of protein, which suggests that the only protein species present in significant concentration are PA_0 and $Pa\text{-}Ca_2$. The kinetics of calcium binding measured by stopped-flow fluorescence are accurately single exponential from 2×10^{-7} to 2×10^{-4} M free calcium. The kinetics of calcium dissociation show a pronounced lag and are best fit by two rate constants of 1.2 and 3.0 s^{-1}. The minimal kinetic mechanism that adequately describes the rate and equilibrium data is a branched pathway mechanism in which the rate and equilibrium constants are markedly different for each pathway:



At [Ca] < 2 μM the upper kinetic pathway of calcium binding predominates whereas at [Ca] > 2 μM calcium binding occurs predominately by the lower kinetic pathway. Calcium dissociates primarily by the upper kinetic pathway.

Parvalbumins are soluble, acidic calcium binding proteins that occur in high concentrations in fish and amphibian skeletal muscle and somewhat lower concentrations in the muscles of higher vertebrates (Hamoir & Konosu, 1965). The three-dimensional structure of carp parvalbumin has been determined to atomic resolution by X-ray diffraction (Moews & Kretsinger, 1975). Carp parvalbumin is a very nearly symmetrical molecule containing six helices. Two pairs of helices are critical in forming the calcium binding sites on the protein. A comparison of the primary and tertiary structures of carp paravalbumin with those of other calcium binding proteins, calmodulin, troponin C, and the myosin light chains, have indicated common genetic origin and conservation of amino acid sequences of the calcium binding sites (Weeds & McLachlan, 1974). In spite of the wealth of structural information, there is considerable disagreement in the literature about some of the basic features of calcium binding. For carp parvalbumin there are published reports of cooperative (Cave et al., 1979), anticooperative (Donato & Martin, 1974), and independent binding of calcium to the two high-affinity sites (Potter et al., 1977). Part of the variability is likely to be due to experimental difficulties associated with making equilibrium calcium binding measurements to very high affinity sites. In addition, the lack of an intrinsic protein chromophore that is sensitive to calcium binding in carp parvalbumin has prevented spectroscopic binding studies.

Parvalbumin from whiting (*Gadus merlangus*) and several closely related fish contains a single tryptophan residue at amino acid position 102 which has absorption and fluorescence spectra that are exquisitely sensitive to calcium binding (Closset & Gerday, 1975). The calcium-dependent fluorescence properties of whiting parvalbumin have been used to measure the equilibrium constants for calcium binding to be 5×10^8 M^{-1} and 6×10^6 M^{-1} for the first and second molecules of calcium bound, respectively (Permyakov et al., 1980).

The steady-state spectral measurements are extended in this paper to include CaEGTA[1] titrations of the circular dichroism, difference spectra, and fluorescence emission spectra. Rate constants for calcium binding and dissociation have been measured by stopped-flow fluorescence. The combined kinetic and equilibrium measurements enable rate constants to be assigned for each of the calcium sites. The equilibrium values reported here agree quite well with those of Permyakov et al. However, the data reported in this paper indicate that calcium binding is cooperative; that is, the second calcium bound to parvalbumin has a higher affinity than the first. Moreover, the kinetic measurements provide evidence that calcium binding occurs by a branched pathway mechanism.

Materials and Methods

All inorganic salts and buffers were of reagent grade. All solutions were made from twice distilled water, which atomic absorption analysis found to contain less than 5×10^{-7} M

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetate; XTA, generalized calcium chelator (EGTA, EDTA, or NTA); DTT, dithiothreitol; BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; PA_0, $PA\text{-}Ca_1$, and $PA\text{-}Ca_2$, molecular species of parvalbumin with 0, 1, and 2 mol of calcium bound per mole of protein, respectively; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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calcium. For experiments in which calcium was removed stoichiometrically (Figures 1–3), specially purified potassium chloride (Suprapurgrade, EM Laboratories, <0.1 ppm calcium) was used to reduce calcium contamination. Solutions of 20 mM borate–0.1 M KCl contained less than 10^{-6} M calcium when measured by atomic absorption. Whiting (*Gadus merlangus*), purchased from Berwick Street fishmonger, Berwick Street, London, was filleted and twice minced before extraction with 10 mM EDTA–20 mM Tris, pH 8.7, 4 °C for 2 h. The low-speed (2000*g*) supernatant was fractionated with acetone (Rao et al., 1969). Preparative centrifuges such as the Beckman J 2-21 series that operate under partial vacuum should not be used to centrifuge solutions containing acetone due to a risk of fire. The 55–80% acetone (v/v) precipitate contained approximately 75% parvalbumin (based upon analysis by SDS electrophoresis). Higher molecular weight impurities were removed by chromatography on Sephadex G-75 or G-50 in 0.1 M potassium chloride–20 mM Tris, pH 8.0. Preparations giving a single band upon electrophoresis on nondissociating gels (Davis, 1964) were used without further purification. The second more slowly migrating component, which comprised up to 20% of the protein in some preparations, was removed as required by DEAE-cellulose chromatography (Closset & Gerday, 1976). The minor component detected with the nondissociating gel system was probably whiting paravalbumin IIIa, although this was never verified. A molar extinction coefficient of 7400 M^{-1} cm^{-1} at 280 nm was used to determine protein concentration.

Steady-state UV absorption, circular dichroism, and fluorescence spectra were obtained on a Cary 121 spectrophotometer, a Cary 60 spectrophotometer, and a Spex fluorolog or Perkin-Elmer MPA2 spectrofluorometer, respectively. Calcium was removed from parvalbumin by three different procedures: (1) in situ removal of calcium by direct addition of EGTA, (2) removal of calcium by addition of a fourfold molar excess of EGTA relative to parvalbumin (in 0.1 M KCl, 10 mM borate, 1 mM dithiothreitol, pH 9) followed by chromatography on G-25 Sephadex to remove CaEGTA and EGTA, and (3) chromatography of parvalbumin solutions on columns containing 1 mg/mL Ca-free carp parvalbumin covalently cross-linked to Sepharose 6B. Methods 2 and 3 were found to leave less than 0.2 mol of calcium/mol of parvalbumin by atomic absorption measurements. Less than 0.1 mol of [^{3}H]EGTA/mol of whiting parvalbumin remained in calcium-free parvalbumin after procedure 2 or 3. [^{3}H]EGTA was obtained from New England Nuclear.

Standard 1 M calcium chloride was purchased from Sigma and verified by titration of the chloride ion with silver nitrate. Calculations of pK'_{Ca} for EDTA, EGTA, and NTA under experimental conditions were based upon the following literature values in 0.1 M KCl, 20 °C, for pK_{Ca} and pK_a': $HNTA^{2-} \leftrightarrow NTA^{3-} + H$, 9.73 (Moddak & Oertel, 1957); $CaNTA^{1-} \leftrightarrow NTA^{3-} + Ca$, 6.46 (Irving & Miles, 1966); $HEGTA^{3-} \leftrightarrow EGTA^{4-} + H$, 9.46 (Anderegg, 1964); $H_2EGTA^{2-} \leftrightarrow HEGTA^{3-} + H$, 8.85 (Anderegg, 1964); $CaEGTA^{2-} \leftrightarrow EGTA^{4-} + Ca$, 11.0 (Irving & Miles, 1966); $HEDTA^{3-} \leftrightarrow EDTA^{4-} + H$, 10.23 (Grimes et al., 1963); $CaEDTA^{2-} \leftrightarrow EDTA^{4-} + Ca$, 10.59 (Grimes et al., 1963). Apparent pK_{Ca} values at a given pH, pK'_{Ca}, were calculated from the pK_{Ca} of the deprotonated ligand, $pK^0{}_{Ca}$, and the pK_a(s) with eq 1 (Gratzer & Beavens, 1977).

$$pK'_{Ca} = pK^0{}_{Ca} - \log [1 + 10^{pK_{a1}-pH} + 10^{pK_{a1}pK_{a2}-2(pH)}] \quad (1)$$

CaEGTA, CaNTA, and CaEDTA stock solutions were made by titrating EGTA, NTA, and EDTA with stock $CaCl_2$ to the free [Ca^{2+}] concentration calculated from pK'_{Ca}. Direct measurement of the concentration of free Ca ion was made with a radiometer calcium selectrode (previously calibrated with a set of standard calcium solutions from 10^{-1} to 10^{-4} M). The required amount of calcium chloride was always within 2% of the calculated amount. In this way, Ca-XTA solutions could be made in which total calcium accurately equals [XTA]. Calcium buffers of XTA and CaXTA could be made volumetrically for a ratio of [CaXTA]/[XTA] from 0.1 to 10. The pH of the calcium buffers made in this way was constant to within ±0.01 pH unit.

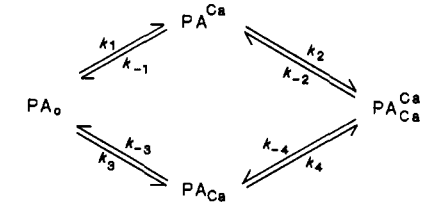
The calculated [Ca] for CaNTA and CaEDTA buffers were in good agreement with calcium selectrode measurements; however, [Ca] in the CaEGTA buffers was ~2 times lower than that calculated from the pK'_{Ca}.

Kinetics of calcium association and dissociation from parvalbumin were made with a stopped-flow fluorometer. Details of the 2 cm × 1 mm cell (dead time ~2 ms) are described elsewhere (White, 1982). Excitation was provided by an Osram 100 W/2 mercury arc lamp equipped with a 290 ± 5 nm interference filter. Emission wavelengths were selected by use of 323 ± 5, 340 ± 5, or 380 ± 5 nm interference filters or a 360-nm hi-pass cutoff filter (Oriel Co.). The photomultiplier output was interfaced to a Data General Nova III minicomputer for data storage and calculations. The data were fit to equations for single or double exponentials with floating end points by the method of moments (Dyson & Isenberg, 1971) or by nonlinear least-squares (Foss, 1970). Calculated fits were compared to the observed data either by plotting of both on the same graph or by direct comparison of printouts of the data. Data were fit with two exponentials only for experiments in which there was a significant improvement in the quality of the fit.

Explicit solutions for two-stepped linear (eq 2), three-stepped linear (eq 3), and branched (eq 4) pathway kinetic mechanisms were obtained with Laplace transforms.

$$PA_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PA\text{-}Ca \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PA\text{-}Ca_2 \quad (2)$$

$$PA_0 \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} PA_0\text{-}Ca \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PA\text{-}Ca_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PA\text{-}Ca_2 \quad (3)$$



(4)

The general solution shown in eq 5a is a sum of exponentials,

$$I(t) = I_0 + \sum_{i=1}^{n-1} I_i e^{-k_i t} \quad (5a)$$

$$I_i = \sum_{j=1}^{n} a_{ji} f_j / \sum_{i=1}^{n-1} \sum_{j=1}^{n} a_{ji} f_j \quad (5b)$$

where I_i are the normalized amplitude coefficients, k_i are the observed rate constants, n is the number of intermediates in the mechanism, and I_0 is the initial fluorescence intensity. The normalized amplitude coefficients, I_i, are calculated with eq 5b from f_j, the changes in the fluorescence emission of each intermediate relative to the first, and a_{ji}, which are functions of the initial concentrations at time zero and the rate constants. The solution to a two-step mechanism (eq 2) obtained by Laplace transforms, the sum of two exponential terms (n = 2, eq 5a), was the same as has been obtained previously by

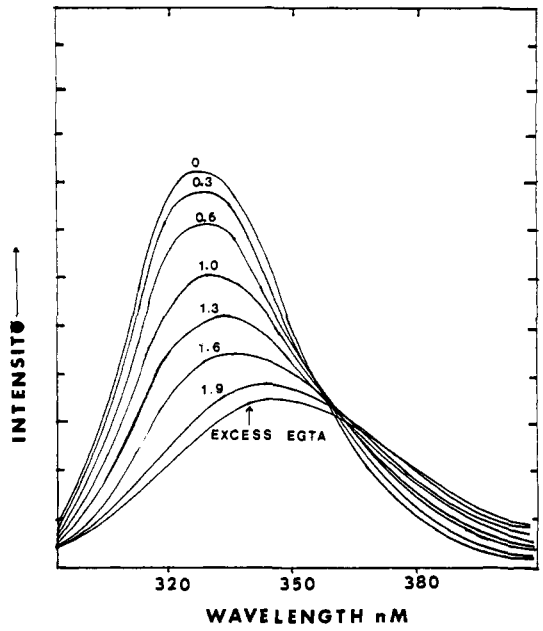


FIGURE 1: Dependence of the fluorescence emission spectra of whiting parvalbumin upon calcium saturation. The cell initially contained 0.2 mg of protein in 0.1 M potassium chloride, 20 mM borate, and 1 mM dithiothreitol, pH 9, 20 °C, in a total volume of 2.0 mL (8.1 μM protein). Calcium was removed in situ by the addition of 10 μL of 0.5 mM EGTA in 0.1 M KCl, pH 9. The spectra were not corrected for dilution of protein. Excitation was at 290 nm with 5-nm bandwidth.

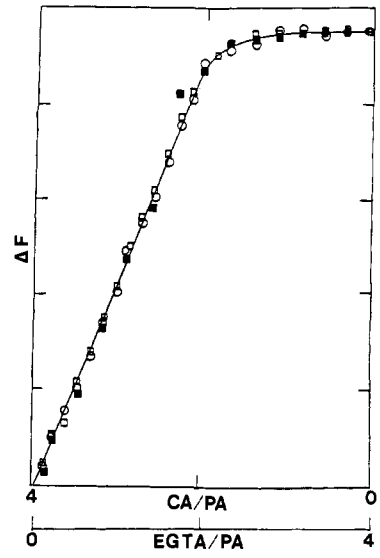


FIGURE 2: Calcium titration of whiting parvalbumin. Experimental conditions are identical with those of Figure 1 except additions were comprised of 5 μL of either 0.5 mM EGTA (□, ■) or 0.5 mM calcium chloride (○); emission is at 325 nm (□, ○) or 380 nm (■). The normalized fluorescence change, F, was calculated from the equation $\Delta F = (F - F_0)/(F_2 - F_0)$, where F_0 and F_2 are the fluorescence of apoparvalbumin and calcium-saturated parvalbumin measured in the presence of 10 mM EGTA and 1 mM $CaCl_2$, respectively.

other methods (Fersht, 1977). Solutions of the three-stepped linear (eq 3) and branched pathway mechanisms (eq 4) are the sums of three exponential terms ($n = 3$, eq 5a). The time dependence of the fluorescence intensity, $I(t)$, for a given set of rate constants, the relative fluorescence intensities, and the free calcium concentrations was obtained from a basic program run on a DEC Rainbow or IBM PC to calculate the normalized amplitude coefficients, I_i, and rate constants, k_i, of eq 5a.

RESULTS

At pH 9 the apparent binding constant of calcium to EGTA is 5×10^{10} M^{-1}, which is approximately 100-fold tighter than the highest reported affinity of calcium to whiting parvalbumin (Permyakov et al., 1980). At pH 9, therefore, EGTA should stoichiometrically remove calcium from whiting parvalbumin. Figure 1 shows the expected stoichiometric removal of calcium from whiting parvalbumin by EGTA. The intensity of the fluorescence emission decreases monotonically, and the emission maxima shifts from 325 to 346 nm. Full calcium removal requires approximately 2 mol of EGTA/mol of parvalbumin. At approximately 360 nm the spectra are very nearly isoemissive over the complete range of calcium saturation. The presence of an isoemissive point suggests that the only two molecular species present in significant concentration during the titration are PA_0 and $Pa\text{-}Ca_2$. Most of the apparent "slippage" of the isoemission point is due to dilution of the sample by addition of the EGTA, which reduces the intensity of the spectra in the presence of 2 mol of EGTA/parvalbumin by 4%.

Qualitatively similar changes have been observed in the fluorescence emission spectrum of whiting parvalbumin by Permyakov et al. (1980) and in the fluorescence emissions spectrum of the cod parvalbumin, which also contains only a single tryptophan residue, by Breen et al. (1985a,b). Both of these laboratories have found deviations from linearity in the increase in fluorescence emission with increasing [Ca]/PA which they have interpreted as evidence for anticooperative calcium binding, $K_I \gg K_{II}$.[2] In order to test this prediction, fluorescence emission was also measured at 380 nm, where there is a decrease in fluorescence emission observed upon calcium binding. The rationale for doing the titration at a second wavelength is that the putative $Pa\text{-}Ca_1$ intermediate might be more easily observed at a different wavelength. The dependence of the normalized spectra at 325 (open symbols) and 380 nm (solid symbols) upon the removal of calcium by EGTA is shown in Figure 2 to be identical at the two wavelengths and to require 1.85 ± 0.1 mol of EGTA/mol of parvalbumin for the maximal change. These data are consistent with models in which either the spectra of the putative $PA\text{-}Ca_1$ are the average of PA_0 and $PA\text{-}Ca_2$ at more than one wavelength or $K_I \ll K_{II}$ and the concentration of $PA\text{-}CA_1$ is low. The presence of exogenous thiol (1 mM dithiothreitol) was required in order to obtain quantitative reversal of the spectral change by the addition of calcium, which is shown by the open circles in Figure 2. In the absence of dithiothreitol, the spectra are only partially reversed by excess calcium (not shown), presumably because of oxidation of cysteine residues which are more reactive in the Ca-free protein. Complete reversal of the spectral change observed by the removal of calcium in the absence of DTT could only be obtained if DTT were added within 1 or 2 h after calcium is removed. After longer periods complete reversal of the spectral changes cannot be obtained. These results are consistent with the observation that whiting parvalbumin has a tendency to form aggregates upon calcium removal in the absence of thiols (Closset & Gerday, 1976).

Equilibrium Measurements of the Affinity of Calcium to Whiting Parvalbumin. The stoichiometric relationship between calcium bound to whiting parvalbumin and the intensity of the fluorescence emission at 325 nm, demonstrated in Figure

[2] K_I and K_{II} are the stoichiometric equilibrium constants for the first and second moles of calcium bound to parvalbumin. They are differentiated from Arabic subscripts for rate and equilibrium constants, K_1 and K_2, which are molecular constants that refer to a specific mechanism or site.

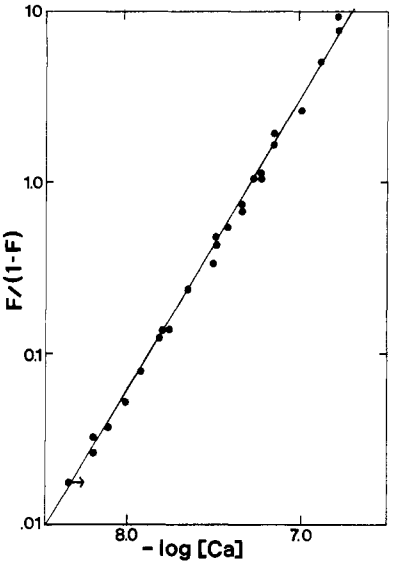


FIGURE 3: Hill plot of calcium binding to whiting parvalbumin. Experimental conditions were 0.1 mg/mL parvalbumin (8.9 μM), 0.1 M potassium chloride, 20 mM Bis-Tris, 1 mM DTT, and either 1 mM EGTA + 1–10 mM CaEGTA or 1 mM CaEGTA + 1–40 mM EGTA, pH 7, 20 °C. The fraction of the parvalbumin sites with calcium bound was obtained from fractional fluorescence emission at 325 nm, which was calculated as described in the legend of Figure 2. The theoretical curve is $K_I K_{II} = 2.0 \times 10^{14}$ M^{-2} with a Hill coefficient of 1.8. The arrow indicates the free calcium concentration that would occur if there were a 0.5% contaminant of Ca in the EGTA and 0.02 mM Ca derived from the parvalbumin.

2, provides a sensitive spectroscopic method to determine the fraction of occupied calcium binding sites. The calcium chelators EDTA, EGTA, and NTA were used to buffer the free calcium concentration so that small errors in the amount of calcium bound to the protein would not result in large errors in the free calcium concentration. An example of this type of experiment is illustrated by a Hill plot in Figure 3. The line drawn through the data is for $K_I K_{II} = 2 \times 10^{14}$ M^{-2} and a Hill coefficient of 1.8. The ratios of [XTA] and [CaXTA] to [PA] were always greater than 100 so that protein-bound calcium would not contribute significantly to total calcium or significantly affect the concentration of free calcium. Atomic absorption measurements indicated that the EGTA and EDTA used in these experiments may contain up to 0.5% calcium. The free calcium concentration in these experiments is most sensitive to errors at the lowest concentrations of free calcium. The error in pCa calculated for possible calcium contamination in the EGTA (0.2 mM) and protein-bound calcium (0.02 mM) is log ([40 mM EGTA]/[1 mM CaEGTA]) − log ([40 − 0.22 mM EGTA]/[1 + 0.22 mM CaEGTA]) = 0.1. The arrow in the lower left hand corner of Figure 2 indicates the magnitude of the error. Between pCa 6.7 and pCa 7.7, such errors are less than 0.04 pCa unit and are not significant. Correction of the pCa for calcium derived from the protein and EGTA would increase the Hill coefficient to 1.9 but not significantly alter the value of $K_I K_{II}$. Direct analysis of spectroscopic data to obtain equilibrium constants for binding of ligand to two sites in a macromolecule can only be made if the Hill coefficient is >1.4 (Grabarek, 1984), a condition observed here for the binding of calcium to whiting parvalbumin. Equilibrium experiments were also done with two other calcium buffers, EDTA and NTA. The differences in $K_I K_{II}$ obtained between experiments using EGTA and EDTA or NTA shown by the data in Table I are most likely due to experimental error and errors in the pK_a and pK_{Ca} values used to calculate free calcium concentration.

Table I: Overall Equilibrium Values for Calcium Binding to Whiting Parvalbumin[a]

$$PA_0 \overset{K_I}{\rightleftharpoons} PA\text{-}Ca_1 \overset{K_{II}}{\rightleftharpoons} PA\text{-}Ca_2$$

XTA[b]	no. of expt	$K_I K_{II}$ (M^{-2})	n
2–10 mM EGTA	5	$(1.5 \pm 0.3) \times 10^{14}$	2.0 ± 0.2
0.2–1.0 mM EGTA	2	$(2.0 \pm 0.3) \times 10^{14}$	1.7 ± 0.2
2–10 mM EDTA	3	$(4.0 \pm 1.0) \times 10^{13}$	1.9 ± 0.4
2–10 mM NTA	3	$(8.0 \pm 2.0) \times 10^{13}$	2.1 ± 0.2

[a] Equilibrium constants K_I and K_{II} are the stoichiometric equilibrium constants for the first and second calcium atoms bound to whiting parvalbumin. Stoichiometric equilibrium constants are distinguished from microscopic equilibrium rate and equilibrium such as those shown in equations 2–4 for calcium binding to specific sites. [b] Experimental conditions: 0.1 M KCl, 20 mM PIPES, 20 °C, and pH 7 or 0.1 M KCl, 20 mM borate, and pH 9.5 (NTA only).

The almost identical affinity of whiting parvalbumin for calcium at pH 7 and pH 9.5 suggests that there is little if any dependence of the binding upon pH in the range 7–9.5. This is in sharp contrast to the calcium chelators, EGTA, EDTA, and NTA, for which calcium binding is very pH dependent. The lack of an observed pH dependence of calcium binding to whiting parvalbumin can be attributed to the absence of ligands to the calcium having pK_a in this range and an absence of titratable groups in the protein with pK_a between 7 and 9.5. The sulfhydryl residues which are quite distant from the calcium binding site are the only groups with pK_a in the region 7.0–9.5. The apparent decrease in reactivity of enzyme sulfhydryl groups observed upon calcium binding could be attributed to thermodynamic linkage between calcium binding and sulfhydryl ionization. However the lack of a pH dependence of the rate and equilibrium constants of calcium binding suggests that the decrease in sulfhydryl reactivity is kinetic rather than thermodynamic.

Calcium Dependence of the Difference Spectra and Circular Dichroism. Both the near-ultraviolet circular dichroism and absorption spectra of whiting parvalbumin are dramatically changed by the removal of calcium (Closset & Gerday, 1976). The spectral changes were investigated at partial calcium occupancy in an attempt to see if a spectrally distinct $PA\text{-}Ca_1$ not observed by fluorescence measurements could be detected. The difference spectra are primarily due to a blue shift in the tryptophan absorption upon calcium binding. The three isosbestic points at 284, 290, and 294 nm (Figure 4A) are evidence that only two molecular species are present when the sites are partially saturated and indicate that there is not a significant concentration of a third component such as $PA\text{-}Ca_1$ with a unique spectrum. The near-ultraviolet circular dichroism spectra (Figure 4B) show a decrease in the region from 260 to 300 nm upon the removal of calcium. The spectra at intermediate degrees of calcium saturation appear to be linear combinations of the spectra of PA_0 and $PA\text{-}Ca_2$ and give no indication of significant concentrations of an additional component such as $PA\text{-}Ca_1$ with a unique spectra.

Kinetics of Calcium Dissociation from Parvalbumin. The kinetics of calcium dissociation from parvalbumin, measured with the change in intrinsic tryptophan fluorescence observed upon mixing $PA\text{-}Ca_2$ with EGTA, are illustrated in Figure 5. The fluorescence change is not first order but has a pronounced lag phase. The data are poorly fit by a single exponential equation, as shown by the dashed line through the data, but are fit extremely well by the sum of two exponentials (eq 5a, $n = 2$) with rate constants of 1.2 and 3.0 s^{-1} as shown by the solid lines through the data. The lag in the observed fluorescence decrease is characteristic of a sequential two-step mechanism in which most of the spectroscopic change occurs

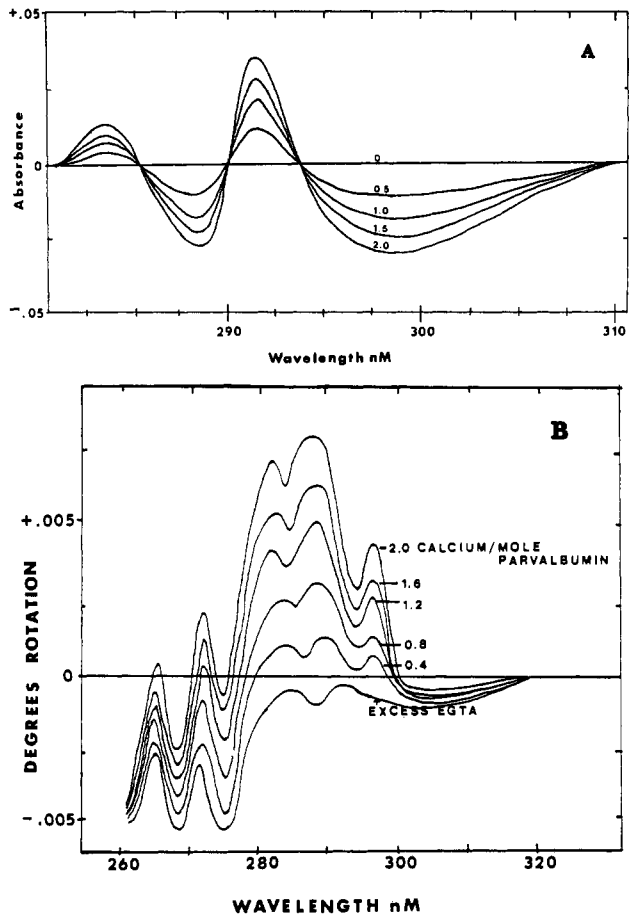


FIGURE 4: Difference and circular dichroism spectra obtained upon calcium binding to whiting parvalbumin. (A) Sample and reference cells initially contained 1 mg of whiting parvalbumin from which calcium and EGTA had been removed as described under Materials and Methods and 0.1 M potassium chloride, 1 mM dithiothreitol, and 20 mM borate, pH 9, 20 °C, in a volume of 2.0 mL (44.5 μM protein). Additions of 10 μL of 5 mM calcium chloride were made to the sample and buffer in the reference cell. (B) Experimental conditions were the same as in (A) except that 10-μL additions of EGTA were made to a 1-cm cell containing 2.5 mL of protein (35.6 μM).

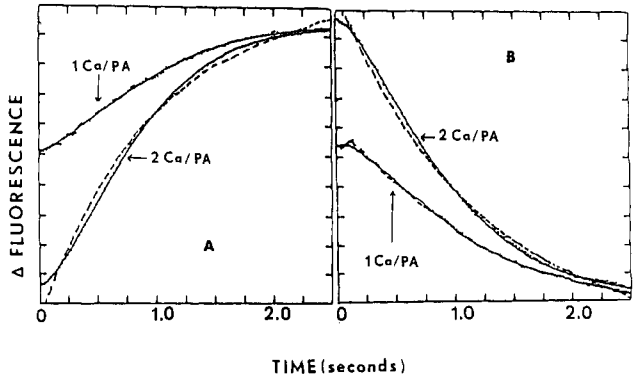


FIGURE 5: Kinetics of calcium dissociation from parvalbumin. In (A) and (B) one syringe of the stopped-flow fluorometer contained 0.1 mg/mL parvalbumin, 0.1 M KCl, 20 mM borate, 10 mM dithiothreitol and either 0 (large-amplitude signals) or 10 μM EGTA (small-amplitude signals). The second syringe contained 10 mM EGTA in the same buffer. Emission was measured at either >360 (A) or 324 nm (B). Solid lines through the data points are theoretical curves derived from computer fits of the data to two exponentials (eq 5a, $n = 2$). Attempts to fit the data with a single exponential are shown by a dashed lines (– –).

with the second step (Fersht, 1977). The normalized amplitude coefficients, I_1 and I_2, which best fit the data for the dissociation of calcium from whiting parvalbumin are 1.7 ± 0.2 for 1.2 s^{-1} and −0.7 ± 0.2 for 3 s^{-1}. The expressions for the normalized amplitude coefficents for the irreversible two-step reaction given in eq 6a and 6b were derived from eq

$$I_1 = -\frac{f_{PA\text{-}Ca_1}}{f_{PA\text{-}Ca_2}}\frac{k_{-2}}{k_{-1}-k_{-2}} + \frac{k_{-1}}{k_{-1}-k_{-2}} \quad (6a)$$

$$I_2 = \frac{f_{PA\text{-}Ca_1}}{f_{PA\text{-}Ca_2}}\frac{k_{-2}}{k_{-1}-k_{-2}} - \frac{k_{-2}}{k_{-1}-k_{-2}} \quad (6b)$$

5b and the expressions obtained for a_i with Laplace transforms. The same expressions are obtained from the solution of Fersht (1977) for a two-step irreversible mechanism. If there is no fluorescence change associated with the dissociation of the first calcium ($f_{PA\text{-}Ca_1}$ in eq 6a and 6b is 0), then for $k_{-2} = 3.0$ s^{-1} and $k_{-1} = 1.2$ s^{-1}, $I_1 = 1.67$ and $I_2 = -0.67$, and for $k_{-2} = 1.2$ s^{-1} and $k_{-1} = 3.0$ s^{-1}, I_1 −0.67 and $I_2 = 1.67$. These values are within the experimental error of the measured values of 1.7 ± 0.2 and −0.7 ± 0.2, and the observed dependence of the fluorescence intensity upon time is independent of the order of the two rate processes. Normalized amplitude coefficients with opposite signs do not necessarily imply that there are changes in emission with opposite signs but may occur for the first step of an irreversible two-step sequential reaction if there is only a small change in emission associated with the first process. The mathmatical form of a "lag" is the sum of two exponential terms with amplitude coefficients of opposite sign. If all of the fluorescence change were associated with the first calcium removed ($f_{PA\text{-}Ca_1} = f_{PA\text{-}Ca_2}$ in eq 6a and 6b), then $I_2 = 0$ and fluorescence intensity would be single exponential. If the fluorescence change produced from the dissociation of each calcium were the same ($f_{PA\text{-}Ca_1} = 0.5 f_{PA\text{-}Ca_2}$), then by use of eq 6a and 6b for values of $k_{-2} = 3$ s^{-1} and $k_{-1} = 1.2$ s^{-1}, $I_1 = 0.83$ and $I_2 = 0.17$, and for $k_{-2} = 1.2$ s^{-1} and $k_{-1} = 3$ s^{-1}, $I_1 = 1.33$ and $I_2 = -0.33$. In this case the values of I_1 and I_2 are dependent upon the order of the rate constants. Mechanisms in which the fluorescence change is associated with either the first calcium bound or is the same for the first and second calcium atoms are therefore inconsistent with the observed amplitude coefficients. The error limits on the data would, however, be consistent with as much as 20% of the fluorescence change occurring with the dissociation of the first calcium but are inconsistent with the fluorescent change occurring equally with each calcium. The lag phase in the reaction observed here can only be explained by a sequential two-step mechanism, shown in eq 2, in which more than 80% of the fluorescence change occurs with the removal of the second calcium ion. Thus, the linear increase in fluorescence enhancement observed in Figure 2 would only be expected to occur if the second calcium binds with a higher affinity than the first; that is, $K_{II} \gg K_I$.

The kinetics of calcium dissociation measured in Figure 5 do not allow the order of the 1.2 and 3.0 s^{-1} processes to be determined because the change in fluorescence occurs only with the dissociation of the second calcium ion. As shown above, the solution to the kinetic equation is symmetric and the dependence of the fluorescence intensity upon time is independent of the order of the fast and slower steps. However, if the emission intensity of the transient PA-Ca_1 intermediate were known and not zero, it would then be possible to determine the order of the two rate processes. Dissociation of calcium from whiting parvalbumin may also be measured with the fluorescent calcium indicator Quin2. The amplitude of the fluorescence increase due to each calcium atom transfered from parvalbumin to Quin2 is the same for both parvalbumin calcium sites, but the rate is dependent upon the rate that the

calcium dissociates from parvalbumin (eq 7). Binding of

$$\begin{array}{ccccccc} \text{PA-Ca}_2 & \xrightarrow{k_{-2}} & \text{PA-Ca}_1 + \text{Ca} & \xrightarrow{k_{-1}} & \text{PA} + \text{Ca} & & (7) \\ & & + & & + & & \\ & & \text{Quin2} & & \text{Quin2} & & \\ & & \downarrow & & \downarrow & & \\ & & \text{Quin2}^*\text{-Ca} & & \text{Quin2}^*\text{-Ca} & & \end{array}$$

calcium to Quin2 occurs within the mixing time of the stopped flow. The increase in Quin2 fluorescence that occurs upon mixing PA-Ca_2 with calcium-free Quin2 is biphasic and is best fit by the *sum* of two exponential terms with rate constants of 1.2 s^{-1} and 3.0 s^{-1} as shown by the data in the bottom line of the upper section of Table II. These data should be compared with the *difference* between two exponential terms that was observed in the remainder of Table II for tryptophan fluorescence. The order of the dissociation of the calcium from the slow and fast sites can be determined from the amplitudes of the signals obtained with Quin2 because the change in fluorescence intensity is only dependent upon the properties of the Ca–Quin2 complex and must be the same for calcium derived from each site. As can be seen from the second and third rows in the lower half of Table II, the order of the fast and slow processes affects the observed amplitudes of each exponential term because the amplitude coefficients also contain terms that include rate constants as are shown in eq 6a and 6b. The observed amplitude coefficients here indicate that the first calcium dissociates at 3.0 s^{-1} and the second at 1.2 s^{-1}.

The kinetics of the fluorescent changes are independent of EGTA or EDTA concentration from 25 μM to 10 mM EGTA as is shown in Table II. It is important to demonstrate that the kinetics of calcium dissociation are independent of chelator and chelator concentration and, therefore, measure the rate of calcium dissociation from parvalbumin rather than rate-limiting calcium binding to the chelator or chelator binding to the protein.

Similar rate constants, 1.1 and 5.9 s^{-1}, have been measured for the dissociation of calcium from the p*I* 4.75 isozyme of cod parvalbumin from the kinetics of the decrease in tryptophan fluorescence that was observed upon mixing the calcium-saturated protein with the chelator 1,2-diaminocyclohexanetetraacetic acid (Breen, 1985b). Cod parvalbumin, p*I* 4.75, is phylogenetically closely related to whiting parvalbumin, but the tryptophan is in position 108 of the amino acid sequence in cod parvalbumin and position 102 of whiting parvalbumin. The decrease in fluorescence observed upon removal of calcium from cod parvalbumin does not have a "lag" and was best fit by two exponentials having coefficients with the same sign. The difference between the two types of kinetic behavior is important and may not only be an indicator of sequence differences but also different mechanisms of calcium binding. The "lag" observed in the work reported here can only be observed if there is a much smaller fluorescence enhancement associated with the removal of the first calcium atom than that associated with the second. The lag and the linear dependence of fluorescence upon the amount of calcium bound are strong evidence that calcium binds to the second binding site of whiting parvalbumin with a higher affinity than to the first. If the second calcium bound with equal or lower affinity than the first, then a break would be expected in spectroscopic properties such as fluorescence emission and difference spectra at 1 Ca/PA. Such a break was observed by Breen et al. in difference spectra titration of cod parvalbumin at 1 Ca/PA. These results indicate that there are important differences between the mechanisms of calcium binding to whiting and cod parvalbumin.

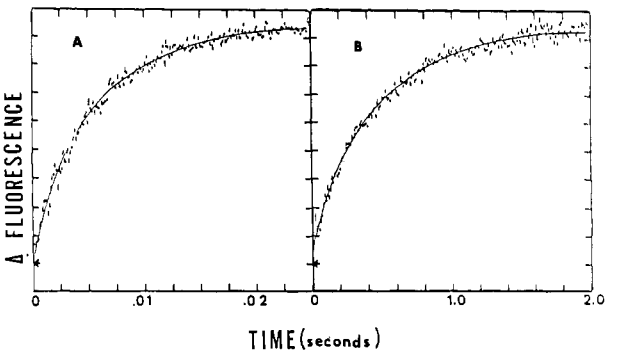


FIGURE 6: Kinetics of calcium binding to whiting parvalbumin. One syringe of the stopped-flow fluorometer contained 0.1 mg/mL whiting parvalbumin, 40 μM EGTA, 20 mM borate, 0.1 M KCl, and 1 mM dithiothreitol, pH 9.0, 20 °C. The second syringe contained either (A) 400 μM $CaCl_2$ or (B) 3 mM NTA and 7 mM CaNTA ([Ca] = 0.81 μM) and 20 mM Bis-Tris and 0.1 M KCl, pH 9. The solid line through the data represents a best fit to the equation $I(t) = I_1 e^{-k_{obsd}t} + I_0$, with (A) k_{obsd} = 255 s^{-1} and (B) k_{obsd} = 3.5 s^{-1}. The arrows show the fluorescence intensity measured upon mixing the protein with buffer.

A further test of the calcium binding mechanism was made by measuring the kinetics of calcium removal after half of the calcium had been removed by 1 mol of EGTA/mol of parvalbumin at pH 9 as shown by the smaller amplitude signals in Figure 5, labeled 1 Ca/Pa. The fluorescence changes follow an identical time course beginning with either completely filled calcium sites or half-saturated sites. The only difference observed was that the amplitude of the change decreased by a factor of 2 if half of the sites were filled. If a significant concentration of PA-Ca_1 were present at half-calcium saturation, it would be expected to have different kinetics of calcium dissociation than PA-Ca_2.

For mechanisms of cooperative calcium binding ($K_{II} \gg K_I$), removal of half of the calcium would leave a one-to-one mixture of PA-Ca_2 and PA_0. Removal of the calcium from the remaining PA-Ca_2 would be expected to have the same rate constants as removal from a solution containing only PA-Ca_2. For alternative binding mechanisms in which the first calcium is bound more tightly than the second, $K_I \gg K_{II}$, removal of one calcium would be expected to produce a solution containing primarily $PACa_1$. Removal of calcium from PA-Ca_1 would be expected to be a single-exponential process rather than the two-exponential kinetics observed here for the removal of the second half of the protein-bound calcium from whiting parvalbumin. The results observed in Figure 5 are readily explained if $K_{II} \gg K_I$ and are not consistent with $K_I \gg K_{II}$. The kinetics of the dissociation of calcium from PA-Ca_2 were measured at three emission wavelengths (323, 340, and 380 nm) to determine whether a contribution from a putative PA-Ca_1 species could be detected in any part of the emission spectra. The results summarized in Table II indicate that the observed kinetic constants of calcium dissociation are independent of emission wavelength and site occupancy.

Kinetics of Calcium Binding to Whiting Parvalbumin. Figure 6 shows the change in fluorescence observed upon mixing calcium-free whiting parvalbumin with a CaNTA buffer having [Ca] of 0.81 μM and with 400 μM $CaCl_2$ in a stopped-flow fluorometer. The increase in fluorescence emission is fit well by a single rate constant over the entire range of calcium concentrations from 0.2 to 250 μM free calcium. The amplitude of the observed fluorescence change is independent of [Ca] from 1 to 250 μM. At [Ca] = 0.2 μM, the amplitude was 60% of that observed at higher concen-

Table II

(A) Summary of Kinetics of Calcium Dissociation from Whiting Parvalbumin[a]

$$PA^*\text{-}CA_2 \overset{k_{-2}}{\rightleftharpoons} PA^*\text{-}CA_1 \overset{k_{-1}}{\rightleftharpoons} PA_0$$

CA/PA	XTA	emission (nm)	k_{-1} (s^{-1})	I_1	k_{-2} (s^{-1})	I_2
2	1 mM EDTA	380	1.4	2.0	2.7	−1.0
1	1 mM EGTA	380	1.2	2.1	2.3	−1.1
2	1 mM EGTA	323	1.4	1.8	3.1	−0.8
1	1 mM EGTA	323	1.0	1.5	3.1	−0.5
2	1 mM EGTA	340	1.3	1.7	3.3	−0.7
1	1 mM EGTA	340	1.3	1.5	2.9	−0.5
2	0.1 mM Quin2	500	1.4	0.7	3.6	0.3

(B) Summary of Modeling of Dissociation Kinetics[b]

f_{PA_0}	$f_{PA\text{-}Ca_1}$	$f_{PA\text{-}Ca_2}$	k_{-1} (s^{-1})	I_1[c]	k_{-2} (s^{-1})	I_2[c]
0.0	1.0	1.0	3.0	−0.7	1.2	1.7
0.0	1.0	1.0	1.2	1.7	3.0	−0.7
0.0	0.5	1.0	3.0	−0.3	1.2	1.3
0.0	0.5	1.0	1.2	0.8	3.0	0.2
0.0	0.0	1.0	3.0	0.0	1.2	1.0
0.0	0.0	1.0	1.2	0.0	3.0	1.0

[a] Experimental conditions: 100 mM KCl, 10 mM Bis-Tris, 20 °C, and pH 9. [b] $I(t) = I_1e^{-k_{-1}t} + I_2e^{-k_{-2}t} + I_0$. [c] Normalized amplitude coefficients, I_1 and I_2, were calculated for the indicated fraction fluorescence amplitudes and rate constants as described under Materials and Methods.

trations. Decreasing the total NTA concentration 10-fold to 1 mM while maintaining the same CaNTA/NTA ratio did not affect the observed rate. The lack of any observable dependence of rate on total NTA concentration must be established in order to show that dissociation of calcium from NTA is not limiting the rate of calcium binding to parvalbumin. Initial experiments in which calcium was removed by incubation with excess 10 mM EGTA at pH 7 and followed by lengthy dialysis to remove the EGTA gave slow nonexponential and poorly reproducible fluorescence kinetics upon mixing the protein with either $CaCl_2$ or CaNTA buffers. Reproducible kinetics of calcium binding could only be obtained if the protein was kept in the presence of 1 mM DTT after calcium was removed. Even in the presence of 1 mM DTT, prolonged storage of whiting parvalbumin in the absence of bound calcium resulted in irreversible spectral changes.

Figure 7 shows a linear dependence of k_{obsd} upon free calcium concentration at free calcium concentrations greater than 5×10^{-6} M. The slope, 1.2×10^6 M^{-1} s^{-1}, is the second-order rate constant for calcium binding to one of the sites. The second-order rate constant of calcium binding to the second site cannot be measured directly but may be estimated with eq 8a and 8b from the overall equilibrium measured in

$$K_IK_{II} = k_1k_2/k_{-1}k_{-2} = 2 \times 10^{14}\ M^{-2} \quad (8a)$$

$$k_2 = (2 \times 10^{14})(k_{-1}k_{-2}/k_1) \sim 6 \times 10^8\ M^{-1}\ s^{-1} \quad (8b)$$

Figure 3 and the three rate constants obtained from kinetic measurements. The simplest kinetic mechanism that is consistent with the data presented so far is a sequential mechanism in which the first calcium binds with a rate constant of 1.2×10^6 M^{-1} s^{-1} and the second calcium binds with a rate expected for diffusion-limited binding of calcium to a protein, $>10^8$ M^{-1} s^{-1}. The apparent second-order rate constant for calcium binding to the second calcium binding site, 6×10^8 M^{-1} s^{-1}, is similar to the second-order rate constant for calcium binding to Quin2, 7.5×10^8 M^{-1} s^{-1}, that can be calculated from the rate and equilibrium constants of calcium dissociation from Quin2 measured by Bayley et al. (1984).

More than 80% of the fluorescence enhancement (indicated by asterisks) is associated with the first calcium bound:

$$PA + Ca \underset{1.2\ s^{-1}}{\overset{1.2 \times 10^6\ M^{-1}\ s^{-1}}{\rightleftharpoons}} PA^*\text{-}CA \underset{3.0\ s^{-1}}{\overset{6 \times 10^8\ M^{-1}\ s^{-1}}{\rightleftharpoons}} PA^*\text{-}Ca_2 \quad (9)$$

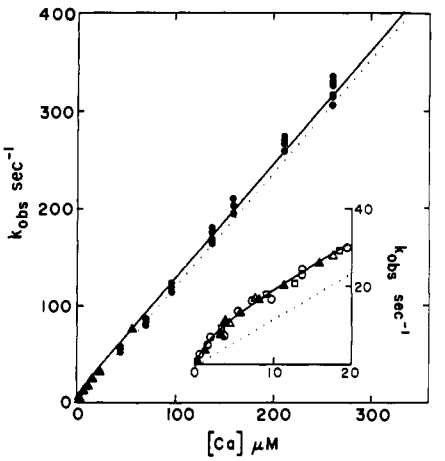
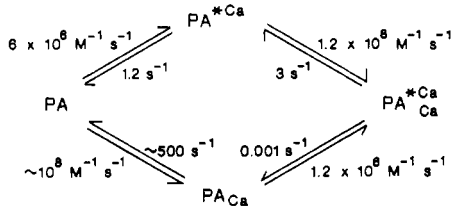


FIGURE 7: Dependence of k_{obsd} on [Ca]. Pseudo-first-order rate constants for calcium binding were obtained in experiments identical with those in Figure 6 except that pH was 7 (△, ●), 8 (□), or 9 (△, ○) and Ca NTA/NTA and hence free [Ca] were varied as indicated. In experiments in which the final calcium concentration was greater than 50 μM, free calcium chloride was used instead of the calcium buffer NTA (●). The dependence of the observed rate constant upon free calcium at calcium concentration less than 20 μM is shown in the insert. The theoretical curves drawn through the data are the rate constants calculated for the predominant (>95% of the amplitude) k_{obsd}. The solid line through the data was calculated for the random calcium binding mechanism with interacting sites of eq 10. The dotted line is for the sequential calcium binding mechanism of eq 9.

The general solution of a two-step binding mechanism is the sum of two exponentials. The dependence of the rate constants and amplitude coefficients of the mechanism shown in eq 9 upon calcium concentration was evaluated with a computer program as described under Materials and Methods. The slower of the two rate processes was found to account for more than 95% of the fluorescence amplitude over the entire concentration range of calcium used (0.2–250 μM). Thus the data would be fit accurately by a single-exponential equation as has been observed. A series of alternative mechanisms have been systematically evaluated in which the order of the rate constants and/or the fluorescence enhancement has been changed are described in the supplementary material (see paragraph at end of paper regarding supplementary material). The observations that the kinetics of calcium binding are fit by a single exponential and that calcium dissociation has a lag phase are very restrictive and eliminate mechanisms in which the

first calcium binds at a diffusion-limited rate and mechanisms in which more than 5% of the fluorescence enhancement is associated with binding of the second calcium. Three-step binding mechanisms in which calcium binds first to form a collision complex that is converted to a more tightly bound intermediate were found to have an almost identical dependence of rate and amplitude upon calcium concentration as the corresponding two-step mechanisms if $k_0k_1 = 1.2 \times 10^6$ M^{-1} s^{-1}.

Although the minimal mechanism shown in eq 9 predicts the observed first-order dependence of the observed rate of calcium binding upon calcium concentration, the dependence of rate upon calcium concentration does not fit the model at [calcium] $< 5 \times 10^{-6}$ M. As shown in the insert to Figure 7 at lower calcium concentrations, the observed rate has a higher dependence upon calcium concentration and extrapolates to ~ 0 s^{-1} at zero calcium concentration. This unusual dependence of rate upon calcium concentration was not predicted by any of the sequential mechanisms tested, which had up to four intermediates. However, a branched-pathway mechanism with the rate constants shown in eq 10 was found



(10)

to produce the observed dependence of the rate upon calcium concentration as shown by the solid line through the data in the insert to Figure 7. The asterisk (*) indicates an enhanced fluorescence emission spectra relative to PA_0 at 325 nm. At [Ca] < 2 μM, the upper pathway predominates the reaction, and the apparent second-order rate constant of calcium binding is limited by the first atom bound at 6×10^6 M^{-1} s^{-1}. The rates of calcium dissociation are those directly measured in Figure 6, and the apparent second-order rate constant for calcium binding to the second site is calculated from the three rate constants and overall equilibrium as was done for the two-step sequential model with eq 8. At [Ca] > 2 μM, the lower pathway is predominant, and the apparent second-order rate constant is that of the second calcium bound, 1.2×10^6 M^{-1} s^{-1}. The value for the equilibrium constant for calcium binding to the first site of the lower pathway, 2 μM, is determined by the best fit to the transition between the upper and lower pathways, which is experimentally illustrated by the change in the second-order rate constant from 6×10^6 to 1.2×10^6 M^{-1} s^{-1} in the insert to Figure 7. Although the rate constants for the binding of the first calcium by the lower pathway cannot be determined independently, a minimum value of k_1 of $\sim 10^8$ M^{-1} s^{-1} was required to obtain single-exponential calcium binding from 0.2 to 200 μM calcium. The rate constant for calcium dissociation from $PA\text{-}Ca_2$ by the lower pathway, 0.001 s^{-1}, is calculated from the overall equilibrium constant, the equilibrium constant for calcium binding to the first site, and the second-order rate constant for calcium binding to the second site. At concentrations of free calcium > 2 μM, the intermediate PA_{Ca} is rapidly formed and binds the second calcium with an apparent second-order rate constant of 1.2×10^6 M^{-1} s^{-1}. At [Ca] < 2 μm the concentration of the PA_{Ca} intermediate is too low for the second step to proceed at a kinetically significant rate, and the upper pathway is dominant. Calcium dissociation via the lower pathway is blocked by the slow rate of dissociation of the first calcium, at 0.001 s^{-1} in the lower pathway, and therefore proceeds by the upper pathway.

Modeled equilibrium data from the sequential and branched mechanisms have linear Hill plots with Hill coefficients of 1.7–1.8 in good agreement with the equilibrium data in Figure 3. Anticooperative mechanisms ($K_I > K_{II}$) have nonlinear Hill plots that are inconsistent with the data in Figure 3. The branched pathway model of eq 10 is physically reasonable: one of the two calcium binding sites (the second step in the upper pathway and the first step in the lower pathway) binds calcium at rates expected for a diffusion-limited reaction and induces little if any change in the environment of the tryptophan; calcium binding to the second site (the first step in the upper pathway and the second step in the lower pathway) is slower as would be expected for a reaction that is associated with significant structural changes in the protein.

Discussion

The large blue shift in the fluorescence emission spectra of whiting parvalbumin observed both here and by Closset and Permyakov upon calcium binding indicates a significant change in the environment of the tryptophan. The analogous residue in carp parvalbumin, phenylalanine-102, is buried in the hydrophobic interior of the protein with little apparent access to solvent. The tryptophan emission spectra of calcium-free whiting parvalbumin is very similar to that observed for tryptophan in free solution. It is therefore apparent that the structure of whiting parvalbumin with calcium removed is a more open structure with the tryptophan in a more polar environment, roughly equivalent to water. This view of the structure of apoparvalbumin–calcium is supported by fluorescence lifetime and quenching measurements (Castelli et al., 1988).

The changes in fluorescence, emission, absorption, and circular dichroism spectra observed upon titration of the protein with EGTA all show linear changes between PA_0 and $PA\text{-}Ca_2$ states. These results, while strongly suggesting cooperative calcium binding, would be consistent with other binding schemes, but only under the unlikely circumstances that the putative $PA\text{-}Ca_1$ intermediate had fluorescence, circular dichroism, and absorption spectra that are very nearly the average of those of PA_0 and $PA\text{-}Ca_2$. The kinetics of binding to and dissociation from whiting parvalbumin provide additional evidence for a strong positive interaction between the calcium binding sites. Evidence for cooperative calcium binding to carp has been found from the proton NMR spectra (Cave et al., 1979). The NMR spectra with 1 mol of calcium bound per mole of protein is equivalent to the spectra of a solution containing half PA_0 and half $PA\text{-}Ca_2$. The cooperative binding reported in this paper and by Cave et al. (1979) appears to be in contradiction with experiments in which only one calcium was removed by a 20-fold excess of EGTA at pH 5.8–7.4 (Donato & Martin, 1974). However, in this pH range the affinity of EGTA for calcium (pK'_{Ca} ~ 7.5 at pH 7.4 and ~ 4.3 at pH 5.8) is similar to or less than the affinity of parvalbumin, pK_{Ca} ~ 7.8, and it is therefore not surprising that calcium was not completely removed.

Equal and independent binding of calcium to the two sites has been reported by Haiech et al. (1979) for frog parvalbumin and Potter et al. (1977) for carp with dissociation constants in the range 10^{-8}–10^{-9} M^{-1}. Part of the differences may reflect species variation. The rate of calcium dissociation from frog parvalbumin measured by stopped-flow measurement of tyrosine fluorescence changes is 0.1 s^{-1} (H. White, unpublished data) which may account for the higher affinity of calcium for the frog and carp proteins if the rate of association is similar to that of whiting.

The lanthanide(III) ions, ytterbium and cerium, have been shown to sequentially replace the two calciums from carp parvalbumin by nuclear magnetic resonance methods (Lee & Sykes, 1981; Williams et al., 1984). The order of replacement, however, reflects only the relative affinities of calcium and the lanthanide ions for each site and does not give information regarding cooperativity of binding for either metal ion.

A plateau in k_{obsd} at high [Ca] was not observed. However, the apparent second-order rate constant of 1.2×10^6 M^{-1} s^{-1} and 6×10^6 M^{-1} s^{-1} for calcium binding to one of the sites of whiting parvalbumin is considerably slower than would be expected for a diffusion-limited rate of calcium binding. The large change in the fluorescence spectrum that occurs upon the binding of the first calcium suggests that the lower than diffusion-limited rate constant is due to considerable changes in the protein structure.

The rate constant for calcium binding to whiting parvalbumin is approximately 10 times less than has been measured for the regulatory calcium binding sites of troponin C (Rosenfeld & Taylor, 1985), and the affinity of whiting parvalbumin for calcium is approximately 100 times larger than has been measured for the regulatory sites of troponin C (Potter et al., 1979). The cooperative mechanism of calcium binding, demonstrated here, provides a mechanism by which the rate of calcium binding can be reduced while maintaining a high affinity. This type of kinetic behavior would serve to make it feasible for parvalbumin to function as an intracellular relaxing factor (Gillis et al., 1982; Somlyo et al., 1985). The "in vivo behavior" of parvalbumin is also likely to depend upon the kinetics of magnesium binding, which may further reduce the rate of calcium binding (Cox et al., 1979; Zhong et al., 1987).

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SUPPLEMENTARY MATERIAL AVAILABLE

Summary of kinetic models for calcium binding to whiting parvalbumin (3 pages). Ordering information is given on any current masthead page.

Registry No. Ca, 7440-70-2.

REFERENCES

Anderegg, G. (1964) *Helv. Chim. Acta 47*, 1801–1814.
Bayley, P., Ahlstrom, P., Martin, S. R., & Forsen, S. (1984) *Biochem. Biophys. Res. Commun. 120*, 185–191.
Breen, P. J., Johnson, K. A., & Horrocks, W. D. (1985a) *Biochemistry 24*, 4997–5004.
Breen, P. J., Hild, E. K., & Horrocks, W. D. (1985b) *Biochemistry 24*, 4992–4997.
Castelli, F., White, H. D., & Forster, L. S. (1988) *Biochemistry* (following paper in this issue).
Cave, A., Pages, M., Morin, P., & Dobson, C. M. (1979) *Biochimie 61*, 607–613.
Closset, J., & Gerday, C. (1975) *Biochim. Biophys. Acta 405*, 228–235.
Closset, J., & Gerday, C. (1976) *Comp. Biochem. Physiol., B: Comp. Biochem. 55B*, 537–542.
Cox, J. A., Winge, D. R., & Stein, E. A. (1979) *Biochimie 61*, 601–605.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–437.
Donato, H., & Martin, R. B. (1974) *Biochemistry 13*, 4575–4579.
Dyson, R., & Isenberg, I. (1971) *Biochemistry 10*, 3233–3241.
Fersht, A. R. (1977) *Enzyme Structure and Mechanism*, pp 118–119, Freeman, San Francisco.
Foss, S. D. (1970) *Biometrics 26*, 815–821.
Gerday, C., & Gillis, J. M. (1976) *Proc. Phys. Soc.*, 96P–97P.
Gillis, J. M., Thomason, D., LeFevre, & G. Kretsinger, R. H. (1982) *J. Muscle Res. Cell Motil. 3*, 377–398.
Grabarek, Z., & Gergely, J. (1984) *J. Biol. Chem. 258*, 14103–14105.
Gratzer, W. B., & Beaven, G. H. (1977) *Anal. Biochem. 81*, 118–129.
Grimes, J. H., Huggard, A. J., & Wilford, S. P. (1963) *J. Inorg. Nucl. Chem. 25*, 1225–1238.
Haiech, J., Derancourt, J., Pechere, J. F., & Demaille, J. G. (1979) *Biochemistry 18*, 2752–2758.
Hamoir, G., & Konosu, S. (1965) *Biochem. J. 96*, 85–97.
Irving, H. M., & Miles, M. G. (1966) *J. Chem. Soc. A*, 727–732.
Johnson, J. D., Charlton, S. C., & Potter, J. D. (1979) *J. Biol. Chem. 254*, 3497–3502.
Lee, L., & Sykes, B. D. (1981) *Biochemistry 20*, 1156–1162.
Moddak, W., & Oertel, G. (1957) *Z. Elektrochem. 61*, 1216–1225.
Moews, P. C., & Kretsinger, R. H. (1975) *J. Mol. Biol. 91*, 201–225.
Permyakov, E., Yarmolenko, V., Emelyanenko, V., Burstein, A., Closset, J., & Gerday, C. (1980) *Eur. J. Biochem. 109*, 307–315.
Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. J., & Means, A. R. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. J., Corradino, R. A., Carofoli, E., Kretsinger, R. H., MacLennan, D. H., & Siegel, F. L., Eds.) pp 239–249, Elsevier, New York.
Rao, K. S. P. B., Focant, B., Gerday, C., & Hamoir, G. (1969) *Comp. Biochem. Physiol. 30*, 33–48.
Rosenfeld, S. S., & Taylor, E. W. (1985) *J. Biol. Chem. 259*, 242–251.
Somlyo, A. V., McClellan, G., Gonzales-Serratos, H., & Somlyo, A. P. (1985) *J. Biol. Chem. 260*, 6801–6807.
Weeds, A. R., & McLachlan, A. D. (1974) Nature (*London*) *252*, 646–649.
White, H. D. (1982) *Methods Enzymol. 85*, 698–708.
Williams, T. C., Corson, D. C., & Sykes, B. D. (1984) *J. Am. Chem. Soc. 106*, 5698–5702.
Zhang, X. Z., & White, H. D. (1987) *Biophys. J. 51*, 322a.