

Static and Kinetic Studies on Carp Muscle Parvalbumins

Takayoshi IIO and Yasuhiko HOSHIHARA

Department of Physics, Faculty of Science, Nagoya University,
Chikusa-ku, Nagoya, Aichi 464

Received for publication, February 23, 1984

Fluorescence titration and fluorescence stopped-flow studies were performed on carp muscle parvalbumin components 1, 2, 3, and 5 (the latter three components were modified with a SH-directed fluorescent reagent, dansyl-L-cysteine). Apparent binding constants (K_{app}) of Ca^{2+} to these components decrease in the order of component 2 ($K_{app} = 2.8 \pm 0.9 \times 10^8 M^{-1}$) > component 1 ($K_{app} = 1.25 \pm 0.25 \times 10^8 M^{-1}$) > component 3 = component 5 ($K_{app} = 4.0 \pm 0.5 \times 10^7 M^{-1}$) in 30 mM KCl, 50 mM Na-cacodylate-HCl, pH 7.0 at 20°C. The rate constant of the conformational change of parvalbumin induced by Ca^{2+} binding or removal decreases in the order of component 2 > component 1 > component 5 $\geq$ component 3; that is, component 2 undergoes the fastest conformational change and component 3 the slowest in response to the rapid free Ca^{2+} concentration ($[Ca^{2+}]$) change in the protein solution. The fluorescence titration curves and $[Ca^{2+}]$ -dependences of the rate constants are analyzed by a simple two-state model, (partially unfolded state) $\xrightleftharpoons[k_2]{k_1}$ (folded state). It is shown that the equilibrium constant $K = k_1/k_2$ depends on the second power of $[Ca^{2+}]$, the rate constant k_1 on the first power of $[Ca^{2+}]$ and k_2 on the inverse first power of $[Ca^{2+}]$, respectively.

Parvalbumins are a family of small, water-soluble proteins present in white muscles of lower vertebrates. They bind strongly two Ca^{2+} per protein molecule, and change their conformation upon the removal of bound Ca^{2+} (1–3). Though their physiological function is still unknown, their physicochemical properties have been widely studied because of their similarity in amino acid sequences

to troponin C and calmodulin (4). Among parvalbumins from various sources, carp muscle parvalbumin has been extensively studied. Carp parvalbumin has five components (1, 5), the amino acid sequences of some of them have been determined (1, 6), and a detailed structural analysis of component 3 (PA3) has been made by X-ray crystallography (7, 8). Parvalbumin is divided into three domains, called the AB loop, the CD loop, and the EF loop, in sequence from the NH_2 terminal (7). Each domain consists of approximately one-third of the polypeptide chain and is characterized by an α -helix-random coil- α -helix structure (7). The two Ca^{2+} -binding sites have been

Abbreviations: PA1, PA2, PA3, and PA5, parvalbumin component 1, 2, 3, and 5; PA2*, PA3*, and PA5*, dansyl-L-cysteine-labeled parvalbumin component 2, 3, and 5; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid; pCa, $-\log[Ca^{2+}]_{free}$.

located within the CD and EF loops (7).

PA3 changes its conformation upon the removal of bound Ca^{2+} ; circular dichroism (CD) measurement showed that the helical content reduced from 47% to 39% (3). This conformational change has been also observed by UV-absorbance, fluorescence and NMR measurements (9, 10).

Since PA3 and the other components have a homologous amino acid sequence (1), the other components have been supposed to show the same physicochemical properties as PA3, though no systematic physicochemical studies have been conducted on the family of carp muscle parvalbumins. This paper reports a systematic study on the conformational change of carp parvalbumin induced by the binding and removal of Ca^{2+} by fluorescence titration and fluorescence stopped-flow methods. Since components 2 (PA2), PA3 and component 5 (PA5) have no suitable intrinsic fluorescent chromophore for study, we labeled these components with a fluorescent SH-directed reagent, dansyl-L-cysteine. (These components have one cysteine residue at position 18 in their sequences (1, 5).) For component 1 (PA1), we adopted tyrosine fluorescence as a measure of the conformational change of the protein (PA1 did not react with the SH-directed reagent). Based on the pCa-dependences of the fluorescence intensity and the kinetic rate constant of the conformational change of parvalbumin, we propose a molecular kinetic mechanism of the conformational change of carp parvalbumin due to the Ca^{2+} binding and removal reactions. We also show that the static and kinetic behaviors of carp parvalbumins are different from each other.

EXPERIMENTAL PROCEDURES

Carp was obtained from a fish market in Tsushima City, Aichi. Parvalbumins were prepared according to the method of Pechère *et al.* (5) with one modification: DEAE-Sephadex A-25 instead of DEAE-cellulose was used in the final stage of purification. In the final preparative chromatogram, we obtained PA1, PA2, and PA3 as distinct bands as Pechère *et al.* did (5). But we could not separate components 5a and 5b as two bands; instead, we obtained single band for PA5 as Brandts *et al.* reported (11). The purity of each

component was checked by disc polyacrylamide gel electrophoresis. PA2, PA3, and PA5 were labeled with dansyl-L-cysteine by addition of 7-fold excess of di-dansyl-L-cystine to each protein in 5 mM EGTA and 20 mM KCl, pH 8.5. The reaction mixture was incubated overnight in a cold room (around 8°C). Free dye was removed by gel-filtration on Sephadex G-25 using 20 mM KCl, pH 8.0, as eluent. The molar ratios of bound dansyl-L-cysteine to the proteins were in the range of 0.22–0.58. The Ca^{2+} bound to the protein was removed by gel-filtration on Sephadex G-25 using 5 mM EGTA and 30 mM KCl, pH 8.0, as eluent before use. The remaining Ca^{2+} in the protein thus treated was measured by Iida's method (12). The protein contained less than 0.22 mol of Ca^{2+} per mol of protein. All subsequent experiments were performed in 30 mM KCl, 50 mM Na-cacodylate-HCl pH 7.0 and various concentrations of EGTA and CaCl_2 at 20°C (EGTA concentration was equal to or higher than 4 mM in all experiments). Protein concentrations were in the range of 0.68–1.97 mg/ml. The protein concentration was determined by the biuret method using PA3 as a standard protein, which has a molar extinction coefficient of 1,751 at 259 nm (13). The pCa value of the solution was calculated from the concentrations of EGTA and CaCl_2 added to the solution using the numerical constants of Schwarzenbach *et al.* (14).

Di-dansyl-L-cystine was purchased from Sigma Chemical Company, U.S.A. EGTA was purchased from Dojindo Laboratory, Japan.

Fluorescence spectra were measured with a Hitachi MPF-2A Spectrofluorometer, and CD spectra with a JASCO J-20 spectropolarimeter.

Kinetic pCa-jump experiments were performed with a Union Giken RA-401 stopped-flow spectrofluorometer for both pCa-down and pCa-up changes. In typical pCa-down experiments, parvalbumin or dye-parvalbumin conjugate was incubated in 5 mM EGTA, 30 mM KCl, 50 mM Na-cacodylate-HCl pH 7.0, and mixed with an equal volume of a CaCl_2 solution containing 5 mM EGTA, 30 mM KCl, and 50 mM Na-cacodylate-HCl in the stopped-flow apparatus. (The pH of the latter solution was preadjusted such that the pH of the mixed solution would be 7.0.) Then the time course of the fluorescence intensity change of the protein solution subsequent to the rapid

change of free Ca^{2+} concentration (pCa-jump) was followed with a Tektronix 5103N storage oscilloscope and analyzed in terms of first-order kinetics. In typical pCa-up experiments, parvalbumin or dye-parvalbumin conjugate was incubated in a buffer solution whose pCa-value was far below the transition region observed in the fluorescence titration curve, and mixed with EGTA solution under the same conditions as the pCa-down experiment. In these experiments, the excitation wavelength was 340 nm for dye-parvalbumin conjugate and 280 nm for PA1, and fluorescence emissions at wavelengths above 420 nm for the former sample and above 290 nm for the latter sample were observed. The instrumental dead time of the stopped-flow apparatus was 3.5 ms under the present experimental conditions.

RESULTS

As the pCa value of the protein solution decreased from above 9.5 (in 5 mM EGTA) to 3.0 at pH 7.0, both PA3 and dye-PA3 conjugate (PA3*) showed 25% increase of the absolute value of the molar ellipticity at 222 nm; and PA3* showed 83% in-

crease of the fluorescence intensity of the bound chromophore. Relative fluorescence intensity of PA3* was measured as a function of pCa by titrating PA3* solution containing 5 mM EGTA with 1 M CaCl_2 solution at pH 7.0. Fluorescence of PA3* showed a transitional increase as pCa decreased from 9 to 6 and became constant below pCa 6.0 (Fig. 1). The molar ellipticity of PA3* at 222 nm showed the same transitional change as the fluorescence (Fig. 1). The transition point was at pCa 7.6 in both curves. Thus, we can conclude the conformational change of the dye-parvalbumin conjugate can be monitored by measuring the fluorescence intensity change of the chromophore. Though the molar ratio of the bound chromophore to the protein was 0.22 for PA3* in Fig. 1, the transition curve obtained from the circular dichroism measurement showed single transition point. So we can regard the fluorescence titration curve of the dye-parvalbumin conjugate as the conformational transition curve of the protein.

Fluorescence titration curves of PA1, dye-PA2 conjugate (PA2*) and dye-PA5 conjugate (PA5*) are shown in Fig. 2. Transition points of the fluorescence titration curves are pCa 8.10

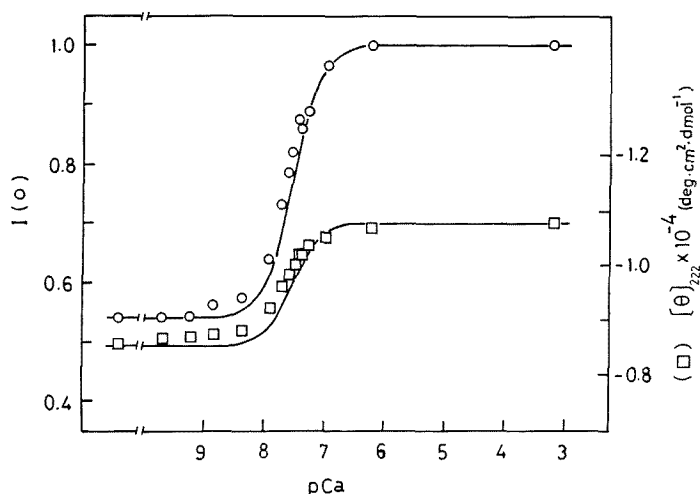


Fig. 1. Change in the relative fluorescence intensity (I) and the molar ellipticity at 222 nm ($[\theta]_{222}$) of PA3* as a function of pCa. Concentration of PA3* was 1.35 mg/ml. Solvent compositions were 30 mM KCl and 50 mM Na-cacodylate-HCl, pH 7.0 at 20°C. pCa of the solution was adjusted with Ca^{2+} -buffer of 5 mM EGTA plus CaCl_2 . Excitation wavelength was 335 nm and emission wavelength 520 nm. The solid lines were calculated curves using Eqs. (4a), (4b), and (5) and numerical constants in Table I.

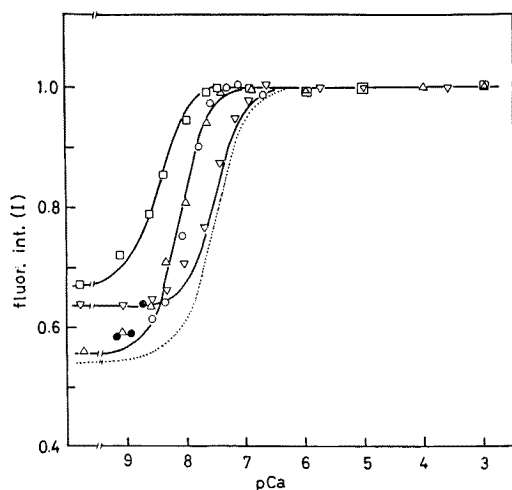


Fig. 2. Fluorescence titration curves of PA1 (Δ), PA2* ($\square$), and PA5* (∇). Experimental conditions were the same as in Fig. 1 except that excitation wavelength for PA1 was 280 nm and emission wavelength 305 nm. Protein concentrations were 1.97 mg/ml for PA1, 1.31 mg/ml for PA2*, and 0.79 mg/ml for PA5*. Symbols $\bullet$ and $\circ$ show the amplitude of the reaction observed in the stopped-flow experiments on PA1: $\bullet$, in pCa-up experiment; $\circ$, in pCa-down experiment. Dotted line shows the fluorescence titration curve of PA3* shown in Fig. 1. Solid lines are the calculated fluorescence titration curves (see text).

for PA1, pCa 8.45 for PA2*, and pCa 7.60 for PA5*.

The time courses of the fluorescence intensity change in all the pCa-jump experiments indicated a pseudo-first-order reaction as typically shown in Fig. 3; that is, $\Delta I(t) = [I(t) - I(t \rightarrow \infty)] = [I(t=0) - I(t \rightarrow \infty)] \times \exp(-kt)$, where $I(t)$ is the fluorescence intensity at time t . The dependence of the amplitude $|I(t=0) - I(t \rightarrow \infty)|$ of the reaction on the final pCa value of the protein solution coincided with the transition curve obtained in the static fluorescence titration experiment (as typically shown in the case of PA1 in Fig. 2). The dependence of the rate constant k on the final pCa value of the protein solution is shown in Fig. 4. (The rate constant did not depend on the pCa value of the protein solution before the pCa-jump experiment.) The rate constant showed a minimum at the transition point of the conformational change of the protein.

The reaction between EGTA and Ca^{2+} is so

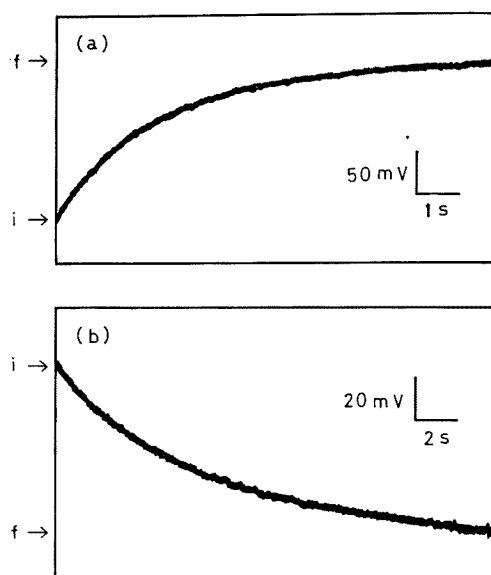


Fig. 3. Oscilloscope tracings of the fluorescence stopped-flow experiment on PA5*. (a) pCa-down experiment from above pCa 9.5 (in 5 mM EGTA) to pCa 6.72, (b) pCa-up experiment from pCa 4.0 to pCa 7.40. Excitation wavelength was 340 nm, emission was observed above 420 nm. Arrows i and f show initial and final levels of the fluorescence intensity. Protein concentration, 1.0 mg/ml. The other experimental conditions were the same as in Fig. 1.

fast as to reach equilibrium within the instrumental dead time under the present experimental conditions (15). Therefore the time course of the fluorescence intensity change subsequent to the pCa-jump was due to the conformational relaxation of the protein toward a new equilibrium state.

In pCa-jump experiments on the mixture of PA2* and PA5*, two phases were observed: one corresponded to the pseudo-first-order reaction of PA2*, the other to that of PA5*. The rate constant of each process was the same as had been observed in the separate experiments on PA2* and PA5*. Thus we can conclude that the kinetic processes of conformational change of carp parvalbumins are independent of each other.

The fluorescence titration and fluorescence stopped-flow experimental results can be analyzed by a simple two-state model:



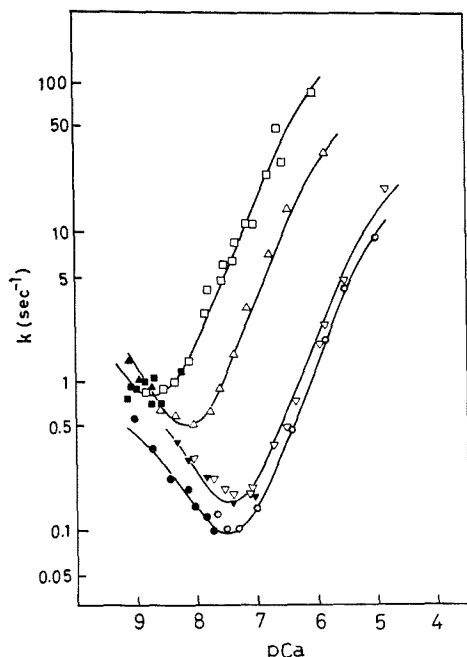


Fig. 4. The rate constant (k) of the conformational change of parvalbumin in 30 mM KCl and 50 mM Na-cacodylate-HCl, pH 7.0 at 20°C. pCa on the abscissa indicates the pCa-value of the mixed solution. Δ , $\square$, k of PA1 (1.48 mg/ml); $\blacksquare$, $\square$, k of PA2* (0.68–1.25 mg/ml); $\bullet$, $\circ$, k of PA3* (0.68–0.91 mg/ml); $\blacktriangledown$, $\triangledown$, k of PA5* (1.0–1.37 mg/ml). Solid symbols show k values obtained from the pCa-up experiments, open symbols the pCa-down experiments. The solid lines are the calculated curves using Eqs. 2, 4a, and 4b and numerical constants in Table I.

where X shows the state of parvalbumin in very low concentration of free Ca^{2+} (high pCa region far from the transition region), Y shows the state of the protein in high concentration of free Ca^{2+} (low pCa region far from the transition region) and k_1 , k_2 are rate constants of the transition between state X and state Y . According to this Scheme I, we can calculate k_1 and k_2 from the equilibrium constant K and the observed rate constant k using the following equations:

$$K = \frac{[Y]}{[X]} = \frac{k_1}{k_2} = \frac{[I(\text{pCa}) - I(5 \text{ mM EGTA})]}{[I(\text{pCa } 3) - I(\text{pCa})]} \quad (1)$$

$$k = k_1 + k_2 \quad (2)$$

where I is the relative fluorescence intensity shown in Figs. 1 and 2.

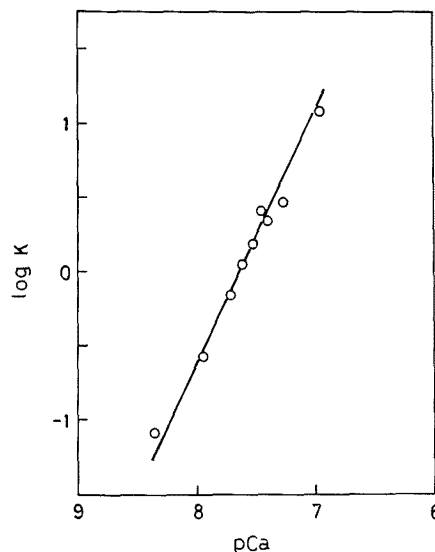


Fig. 5. The equilibrium constant K calculated from the fluorescence titration curve of PA3* in Fig. 1.

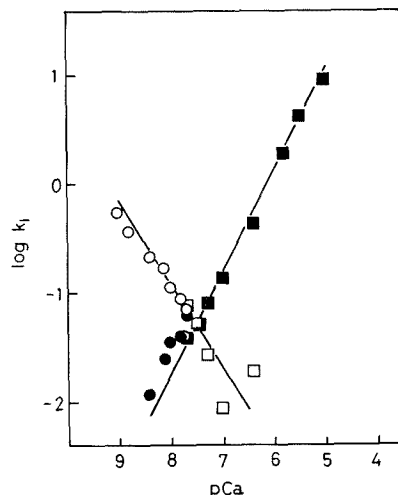


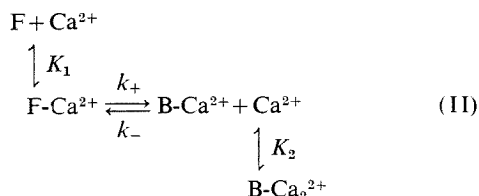
Fig. 6. The rate constants k_1 and k_2 (in s^{-1}) of PA3* in Scheme I (see text). $\blacksquare$ and $\square$, k_1 and k_2 obtained from the pCa-down experiment, respectively; $\bullet$ and $\circ$, k_1 and k_2 obtained in the pCa-up experiment, respectively.

The equilibrium constant K of each parvalbumin component depends roughly on the second power of the free Ca^{2+} concentration ($[\text{Ca}^{2+}]$). As a typical example, the equilibrium constant of PA3* is plotted logarithmically in Fig. 5. The equilibrium constant K of PA3* is proportional to $[\text{Ca}^{2+}]^{1.76}$.

The calculated rate constant k_1 of each parvalbumin component depends roughly on the first power of $[\text{Ca}^{2+}]$, and k_2 on the inverse first power of $[\text{Ca}^{2+}]$. As a typical example, k_1 and k_2 values of PA3* are plotted logarithmically in Fig. 6. It is determined graphically that k_1 of PA3* is proportional to $[\text{Ca}^{2+}]^{0.95}$ and k_2 of PA3* to $[\text{Ca}^{2+}]^{-0.83}$.

DISCUSSION

The findings that the equilibrium constant K depends on the second power of $[\text{Ca}^{2+}]$ (Fig. 5) and that the rate constant k_1 of Scheme I depends on the first power of $[\text{Ca}^{2+}]$ and k_2 on the inverse first power of $[\text{Ca}^{2+}]$ (Fig. 6) have the following implications (16): in the pCa-down experiment, binding of one Ca^{2+} to the partially unfolded Ca^{2+} -free parvalbumin triggers the conformational change to the folded state which binds spontaneously the second Ca^{2+} ; conversely, in the pCa-up experiment, removal of one Ca^{2+} from the folded two Ca^{2+} -bound parvalbumin triggers the conformational change to the partially unfolded state which removes spontaneously remaining bound Ca^{2+} . We can thus describe the molecular kinetic mechanism of the conformational change of parvalbumin by Scheme II:



In this Scheme II, conformational state F is the partially unfolded conformation which parvalbumin assumes in the high pCa region far from the transition region; and parvalbumin can bind one Ca^{2+} in this conformational state. Conformational state B is the folded conformation which parvalbumin assumes in the low pCa region far from the transition region; and parvalbumin binds one or two Ca^{2+} in this conformational state. K_1 and K_2 are the association constants of Ca^{2+} to the F state and B- Ca^{2+} state, respectively. k_+ and k_- are microscopic rate constants of the conformational change between F- Ca^{2+} and B- Ca^{2+} . In the following discussion, we will see that states

F- Ca^{2+} and B- Ca^{2+} are never present in detectable amounts even in the transition region.

Two assumptions are made to explain our static and kinetic experimental results based on Scheme II: (i) The environment of the cysteine residue to which the dye binds, and hence the fluorescence intensity of the conjugated dye, is almost the same in the F and F- Ca^{2+} states. The same is assumed in the case of B- Ca^{2+} and B- Ca_2^{2+} . (ii) Ca^{2+} binding and removal reactions are much faster than the conformational interconversion between F- Ca^{2+} and B- Ca^{2+} ; that is, the interconversion process is the slow step in the overall process.

The X and Y states defined in the two-state model can then be represented as

$$[X] = [F] + [F\text{-Ca}^{2+}] \quad (3a)$$

$$[Y] = [\text{B-Ca}^{2+}] + [\text{B-Ca}_2^{2+}] \quad (3b)$$

k_1 and k_2 in Scheme I can be calculated (17) as

$$k_1 = \frac{k_+ K_1 [\text{Ca}^{2+}]}{1 + K_1 [\text{Ca}^{2+}]} \quad (4a)$$

$$k_2 = \frac{k_-}{1 + K_2 [\text{Ca}^{2+}]} \quad (4b)$$

The pCa-dependence of the fluorescence intensity (fluorescence titration curve) can be calculated from Eqs. 5, 4a, and 4b;

$$\begin{aligned}
 \theta &= \frac{\text{fluorescence increase from the highest pCa region}}{\text{total fluorescence change}} \\
 &= \frac{[Y]}{[X] + [Y]} = \frac{k_1}{k_1 + k_2} \quad (5)
 \end{aligned}$$

and the pCa-dependence of the rate constant k of the conformational change of parvalbumin can be calculated from Eqs. 2, 4a, and 4b.

Numerical values of K_1 , K_2 , k_+ , and k_- in Scheme II are determined graphically by comparing the calculated curves and experimental results shown in Figs. 1, 2, and 4. These numerical constants are listed in Table I. Solid lines in Figs. 1, 2, and 4 are the calculated curves.

The values of K_1 and K_2 in Table I suggest that the states F- Ca^{2+} and B- Ca^{2+} are virtually absent even in the transition region, because $K_1 [\text{Ca}^{2+}] \ll 1$ and $K_2 [\text{Ca}^{2+}] \gg 1$ in this region. Therefore, parvalbumin assumes substantially two states of F and B- Ca_2^{2+} even in the transition region. This is why the transition curves in Figs. 1 and 2

can be analyzed by the two-state model. It is also shown that $k_1 = k_+K_1[\text{Ca}^{2+}]$, $k_2 = k_-K_2^{-1}[\text{Ca}^{2+}]^{-1}$, and $K = (k_+/k_-)K_1K_2[\text{Ca}^{2+}]^2$ in the transition region; these relationships explain the experimental results shown in Figs. 6 and 5.

The binding curve of Ca^{2+} to parvalbumin is calculated as

$$\begin{aligned} \gamma &= \frac{\text{total number of bound } \text{Ca}^{2+}}{\text{total number of parvalbumin}} \\ &= \frac{[\text{F}-\text{Ca}^{2+}] + [\text{B}-\text{Ca}^{2+}] + 2[\text{B}-\text{Ca}_2^{2+}]}{[\text{F}] + [\text{F}-\text{Ca}^{2+}] + [\text{B}-\text{Ca}^{2+}] + [\text{B}-\text{Ca}_2^{2+}]} \\ &= \frac{K_1[\text{Ca}^{2+}] + K_1(k_+/k_-)[\text{Ca}^{2+}] + 2K_1K_2(k_+/k_-)[\text{Ca}^{2+}]^2}{1 + K_1[\text{Ca}^{2+}] + K_1(k_+/k_-)[\text{Ca}^{2+}] + K_1K_2(k_+/k_-)[\text{Ca}^{2+}]^2} \quad (6) \end{aligned}$$

The pCa-dependence of γ gives almost the same curve as the fluorescence titration curve of each parvalbumin shown in Figs. 1 and 2; for instance, the pCa value at which $\gamma = 1$ (midpoint of the Ca^{2+} binding curve) is 8.15 for PA1, 8.45 for PA2*, 7.50 for PA3*, and 7.45 for PA5*.

Based on the three-dimensional structure of the Ca^{2+} -binding sites of PA3, Coffee *et al.* (1) predicted that removal of Ca^{2+} from the EF loop induced the conformational change because one of the ligands in the EF Ca^{2+} -binding site was an oxygen atom of water, and lanthanides were easily displaced from the EF Ca^{2+} -binding site but not from the CD site. Scheme II supports this prediction. We can deduce that, when the free Ca^{2+} concentration of the protein solution is decreased, Ca^{2+} is first removed from the EF Ca^{2+} -binding site of parvalbumin in the B- Ca_2^{2+} state, and that this removal reaction triggers the conformational change from the B- Ca^{2+} state to F- Ca^{2+} state.

The other interesting finding in this work is the difference in the static and kinetic behaviors

of carp parvalbumins: the apparent binding constant of Ca^{2+} , which can be calculated as the inverse of $[\text{Ca}^{2+}]$ at the transition point, is $1.25 \pm 0.25 \times 10^8 \text{ M}^{-1}$ for PA1, $2.8 \pm 0.9 \times 10^8 \text{ M}^{-1}$ for PA2*, and $4.0 \pm 0.5 \times 10^7 \text{ M}^{-1}$ for PA3* and PA5* (Figs. 1 and 2). The rate constant of the conformational change of parvalbumin due to the Ca^{2+} binding and removal reactions decreases in the order of $\text{PA2}^* > \text{PA1} > \text{PA5}^* \geq \text{PA3}^*$ (Fig. 4 and Table I). That is, PA2* binds Ca^{2+} more strongly and undergoes the conformational change more rapidly in response to the change of $[\text{Ca}^{2+}]$ than the other components. PA3* and PA5* show almost the same static and kinetic behaviors; they bind Ca^{2+} more weakly and undergo the conformational change more slowly than the other components.

Though the reason for these differences between the components remains to be solved, some possible factors can be indicated. First, replacements of amino acids in PA2 compared to PA3 are more frequent in the AB loop than in the CD and EF loops (1). Thus the AB loop, which cannot itself bind Ca^{2+} , probably regulates the Ca^{2+} binding capability and the rate of the conformational change of parvalbumin; that is, the AB loop may be a regulatory domain in the parvalbumin protein. Second, the amino acid replacement in the EF helical regions of PA2 favors the α -helical stabilization in this region compared to PA3 and PA5 (1, 18). It is therefore also probable that the Ca^{2+} binding capability and the rate of the conformational change depend on the stability of the α -helices in the EF loop. Alternatively, there may be an unidentified amino acid on which the Ca^{2+} binding capability and the rate of the conformational change of the protein depend.

For the binding constant of Ca^{2+} to PA3, the following values have been determined by the equilibrium dialysis method: $2.7 \times 10^9 \text{ M}^{-1}$ in 25 mM Tricine buffer, pH 7.4, 80 mM KCl and 0.1

TABLE I. Numerical constants in Scheme II.

Sample	$K_1 (\text{M}^{-1})$	$K_2 (\text{M}^{-1})$	$k_+ (\text{s}^{-1})$	$k_- (\text{s}^{-1})$
PA1	4.0×10^5	2.0×10^9	90.0	3.6
PA2*	5.6×10^5	2.7×10^9	240.0	4.0
PA3*	6.2×10^4	3.8×10^8	23.5	0.59
PA5*	7.1×10^4	3.8×10^8	32.0	1.1

mm dithiothreitol at 25°C (19); and $2.8 \times 10^8 \text{ M}^{-1}$ in 0.1 M KCl, 20 mM imidazole, pH 7.5 (20). Our K_{app} value is $4.0 \pm 0.5 \times 10^7 \text{ M}^{-1}$ in 30 mM KCl and 50 mM Na-cacodylate-HCl, pH 7.0, at 20°C. Large discrepancies exist between these values, the causes of which remain for future study.

REFERENCES

1. Coffee, C.J., Bradshaw, R.A., & Kretsinger, R.H. (1974) *Adv. Exp. Med. Biol.* **48**, 211–233
2. Benzonana, G., Capony, J.P., & Pechère, J.F. (1972) *Biochim. Biophys. Acta* **278**, 110–116
3. Donato, H., Jr. & Martin, R.B. (1974) *Biochemistry* **13**, 4575–4579
4. Dedman, J.R., Jackson, R.L., Schreiber, W.E., & Means, A.R. (1978) *J. Biol. Chem.* **253**, 343–346
5. Pechère, J.F., Demaille, J., & Capony, J.P. (1971) *Biochim. Biophys. Acta* **236**, 391–408
6. Coffee, C.J. & Bradshaw, R.A. (1973) *J. Biol. Chem.* **248**, 3305–3312
7. Kretsinger, R.H. & Nockolds, C.E. (1973) *J. Biol. Chem.* **248**, 3313–3326
8. Moews, P.C. & Kretsinger, R.H. (1975) *J. Mol. Biol.* **91**, 201–228
9. Burstein, E.A., Permyakov, E.A., Emelyanenko, V.I., Bushueva, T.L., & Pechère, J.F. (1975) *Biochim. Biophys. Acta* **400**, 1–16
10. Parello, J., Cavé, A., Puigdomenech, P., Maury, C., Capony, J.P., & Pechère, J.F. (1974) *Biochimie* **56**, 61–76
11. Brandts, J.F., Brennan, M., & Lin, L.N. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4178–4181
12. Iida, S. (1979) *J. Biochem.* **86**, 733–743
13. Hoshihara, Y. (1978) M.S. Thesis, Dept. of Phys., Nagoya Univ.
14. Schwarzenbach, G., Senn, H., & Anderegg, G. (1957) *Helv. Chim. Acta* **40**, 1886–1900
15. Harafuji, H. & Ogawa, Y. (1980) *J. Biochem.* **87**, 1305–1312
16. Tanford, C., Pain, R.H., & Otchin, N.S. (1966) *J. Mol. Biol.* **15**, 489–504
17. Iio, T. (1983) *Protein, Nucleic Acid and Enzyme (Bessatsu)* (in Japanese) **26**, 123–133
18. Chou, P.Y. & Fasman, G.D. (1974) *Biochemistry* **13**, 222–244
19. Moeschler, H.J., Schaer, J.J., & Cox, J.A. (1980) *Eur. J. Biochem.* **111**, 73–78
20. 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., Corradino, R.A., Carafoli, E., Kretsinger, R.H., MacLennan, D.H., & Siegel, F.L., eds.) pp. 239–250, North-Holland, New York