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Binding of Calcium by Calmodulin: Influence of the Calmodulin Binding Domain of the Plasma Membrane Calcium Pump†

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ABSTRACT: The interaction between calmodulin and synthetic peptides corresponding to the calmodulin binding domain of the plasma membrane Ca^{2+} pump has been studied by measuring Ca^{2+} binding to calmodulin. The largest peptide (C28W) corresponding to the complete 28 amino acid calmodulin binding domain enhanced the Ca^{2+} affinity of calmodulin by more than 100 times, implying that the binding of Ca^{2+} increased the affinity of calmodulin for the peptide by more than 10^8 times. Deletion of the 8 C-terminal residues from peptide C28W did not decrease the affinity of Ca^{2+} for the high-affinity sites of calmodulin, but it decreased that for the low-affinity sites. A larger deletion (13 residues) decreased the affinity of Ca^{2+} for the high-affinity sites as well. The data suggest that the middle portion of peptide C28W interacts with the C-terminal half of calmodulin. Addition of the peptides to a mixture of tryptic fragments corresponding to the N- and C-terminal halves of calmodulin produced a biphasic Ca^{2+} binding curve, and the effect of peptides was different from that on calmodulin. The result shows that one molecule of peptide C28W binds both calmodulin fragments. Interaction of the two domains of calmodulin through the central helix is necessary for the high-affinity binding of four Ca^{2+} molecules.

The affinity of Ca^{2+} for calmodulin is greatly enhanced by the addition of target enzymes and peptides. This is shown by Ca^{2+} binding studies with enzymes such as myosin light chain kinase and cyclic nucleotide phosphodiesterase (Olwin & Storm, 1985), caldesmon (Yazawa et al., 1987), or the calmodulin-binding peptides melittin (Maulet & Cox, 1983) and mastoparan (Yazawa et al., 1987). Melittin and mastoparan binds strongly to calmodulin in the presence of Ca^{2+} (Maulet & Cox, 1983; Malencik & Anderson, 1983). The studies using mastoparan indicate that communication between the N-terminal and C-terminal domains of calmodulin may play a role in enzyme activation (Yazawa et al., 1987, 1990). This is at variance with what is observed in the target-free, dumbbell-shaped calmodulin molecule (Babu et al., 1988).

The calmodulin binding domain of the plasma membrane Ca^{2+} pump has recently been identified as a 28 amino acid sequence located near the C-terminal portion of the molecule (James et al., 1988; Verma et al., 1988; Vorherr et al., 1990). It shares with other calmodulin-dependent proteins the propensity to form a basic amphiphilic helix (James et al., 1988). Synthetic peptides corresponding to the calmodulin binding domain of the pump have been shown to interact with calmodulin with K_ds in the nanomolar to subnanomolar range (Vorherr et al., 1990). In addition, the peptides inhibit strongly the activity of the Ca^{2+} pump (Enyedi et al., 1989).

In the work presented in this contribution, the interaction between calmodulin and the synthetic peptides corresponding to the calmodulin binding domain of the Ca^{2+} pump was studied by monitoring the binding of Ca^{2+} to calmodulin. A 28-residue peptide (C28W) was found to enhance very significantly the Ca^{2+} binding to calmodulin. The enhancement was also observed with a mixture of two calmodulin fragments, corresponding to the N-terminal and C-terminal halves of the molecule, respectively. However, the Ca^{2+} binding curve of the mixture of the two fragments and peptide C28W became biphasic due to the decreased Ca^{2+} binding to the N-terminal domain. The work underlines the importance of interdomain interaction through the central helix for calmodulin function.

Materials and Methods

Calmodulin was prepared from scallop testis as described previously (Minowa & Yagi, 1984). When it is compared to vertebrate calmodulin, the scallop protein has three conservative substitutions among 148 amino acid residues (Tyr99-Phe, Gln143-Thr, Ala147-Ser) (Toda et al., 1981). Other properties such as Ca^{2+} binding and enzyme activation profiles are the same as for vertebrate calmodulin. Tryptic fragments (F12 and F34) of calmodulin were prepared by digestion in the presence of 2 mM $CaCl_2$ and purified by DEAE-cellulose chromatography as described in a previous paper (Minowa & Yagi, 1984). Fragment F12 (acetyl-Ala1-Lys75) corresponds

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to the N-terminal domain and fragment F34 (Asp78–Lys148) corresponds to the C-terminal domain.

Calmodulin binding peptides were synthesized and purified as described previously (Vorherr et al., 1990). The synthetic peptides used were the following: C28W, LRRGQILWFRGLNRIQTQIKVVNAFSSS; C20W, LRRGQILWFRGLNRIQTQIK; C15, LRRGQILWFRGLNRI; C28A, LRRGQILAFRGLNRIQTQIKVVNAFSSS; C28Y, LRRGQILYFRGLNRIQTQIKVVNAFSSS. Peptide C28W corresponds to the full calmodulin binding domain of the plasma membrane Ca^{2+} pump. Peptide C20W and C15 contain the 20 and 15 N-terminal residues of peptide C28W, respectively. Peptides C28A and C28Y are analogues of peptide C28W with a single substitution (Trp8 for Ala and Tyr, respectively).

Calcium binding to calmodulin and to its fragments was measured by the flow dialysis method of Colowick and Womack with a Reichard-type flow dialysis cell (Womack & Colowick, 1973; Minowa & Yagi, 1984). The experimental medium contained 10–30 μM calmodulin or its fragments, 0.1 M NaCl, 20 mM MOPS–NaOH (pH 7.0)[1] at 25 °C. Glass-distilled and ion-exchanged water with an electric conductivity of 0.06 μS was used throughout the experiments. A Spectrapor 6 cellulose membrane with a molecular weight cutoff of 1000 (Spectrum Medical Industries, Inc., Los Angeles, CA) was used to separate the lower and upper chambers. The flow rate of solvent through the lower chamber was 1 mL/min, and the response time of the apparatus was 4 min. Fractions were collected every 1 min (1 mL/fraction). Immediately before the initiation of the titration, 0.5 mL of the protein solution in the upper chamber was withdrawn to estimate the amount of contaminant Ca^{2+}. The titration was initiated by the addition 3 μM $^{45}CaCl_2$ (Du Pont–NEN, Boston, MA), to 1 mL of the protein solution in the upper chamber. The titration was performed by addition of 5-μL aliquots of different $CaCl_2$ stock solutions to the upper chamber every six fractions. Usually, the final chase-out with 10 mM $CaCl_2$ was done after the 15th cycle of titration. At the end of the titration, less than 8% of $^{45}Ca^{2+}$ was lost from the upper chamber. The calculation of Ca^{2+} binding was corrected for this loss. Under the experimental conditions, the Ca^{2+} binding curves obtained from duplicate experiments were reproducible and almost superimposable with an average Ca^{2+} binding of 3.6 mol/mol of calmodulin around 30 μM free Ca^{2+}, indicating the maximum Ca^{2+} binding of 4 mol/mol. However, the deviations in the experimental data points were fairly large in the very low ranges of free Ca^{2+} concentrations. Even under these limited conditions, the results were analyzed quantitatively in order to understand the characteristics of the interaction between peptides and calmodulin or fragments. Quantitative analyses were performed using a nonlinear regression program to fit the Adair equation (Adair, 1925; Minowa & Yagi, 1984) given by

$$r = (K_1x + 2K_1K_2x^2 + \ldots + nK_1 \ldots K_nx^n)/(1 + K_1x + K_1K_2x^2 + \ldots + K_1K_2 \ldots K_nx^n) \quad (1)$$

where r is the amount of Ca^{2+} bound to calmodulin or its fragments in a molar ratio at free Ca^{2+} concentration x. K_1, K_2, ..., K_n are the macroscopic binding constants, where n corresponds to the maximal number of Ca^{2+} binding sites (n = 4 for calmodulin and n = 2 for fragments F12 or F34). For the purpose of curve fitting, the experimental results were normalized to n = 4 and n = 2, respectively, although the exact values obtained were 3.6 and 1.6 for calmodulin with peptides other than C28W and for the F12 complexes, respectively. The optimally fitted curves are shown in the figures after reduction to the original experimental values corresponding to maximal numbers of Ca^{2+} binding sites. An estimate of the Ca^{2+}-induced increase in the affinity of calmodulin for the peptide was obtained by calculating the ratios of the dissociation constants for the peptide–calmodulin complex from the macroscopic Ca^{2+} binding constants for the peptide–calmodulin complex (K_1', ..., K_4') or for the peptide-free calmodulin (K_1, ..., K_4) from the equation

$$K_d'/K_d = K_1'K_2'K_3'K_4'/K_1K_2K_3K_4 \quad (2)$$

where K_d and K_d' are the dissociation constants for the peptide–calmodulin complex in the Ca^{2+}-saturated and Ca^{2+}-free state, respectively. K_d'/K_d values were calculated in a similar way in the case of the calmodulin fragments.

The concentration of calmodulin was determined spectrophotometrically using an ϵ_{278} = 1620 M^{-1} cm^{-1} (Yazawa et al., 1980). The concentration of fragments F12 and F34 was determined by the biuret method using scallop calmodulin as a standard. Peptide stock solutions were prepared by dissolving known weighed amounts of peptides into H_2O. Contaminant Ca^{2+} in the protein solution, which was less than 0.1 mol/mol by our preparation method using trichloroacetic acid, was determined by the atomic absorption method using a Hitachi 180-30 spectrophotometer.

Results

Ca^{2+} Binding to Calmodulin in the Presence of Peptide C28W and Its Analogues. The binding of Ca^{2+} to calmodulin was measured in the presence of a 1.9-fold molar excess of peptide C28W (30.4 μM) in 0.1 M NaCl and 20 mM MOPS–NaOH (pH 7.0) at 25 °C. As shown in Figure 1, the Ca^{2+} affinity of calmodulin increased upon addition of peptide C28W by more than 100 times as compared to that of target-free calmodulin (dashed line). Cooperativity in the Ca^{2+} binding was not evident in the presence of C28W. Maximal Ca^{2+} binding (3.3 mol/mol in the presence of peptide C28W) was attained at about 30 μM Ca^{2+}. This was lower than other cases giving 3.6 mol/mol (Figures 1 and 2). The increase of the concentration of peptide C28W to 60.8 μM (3.8-fold molar excess) resulted in the decrease of the maximal Ca^{2+} binding to 2.8 mol/mol, without changes in the binding of Ca^{2+} to the high-affinity sites (Figure 1, ×): under these conditions the protein solution in the upper chamber was turbid after the final-chase step. Both results gave a single best-fit curve after normalization of the maximal Ca^{2+} binding to 4.0. Since peptide C28W has a tendency to aggregate at high concentrations (Vorherr et al., 1990), some of the calmodulin in the solution may have precipitated with peptide C28W in a way unable to bind Ca^{2+}. Aside from this effect, peptide C28W strongly enhanced the Ca^{2+} affinity of all four binding sites of calmodulin.

The enhancing effects on the binding of Ca^{2+} to calmodulin were investigated by replacing Trp8 in peptide C28W with Ala8 (C28A) and with Tyr8 (C28Y). Previous work (Vorherr et al., 1990) has shown that these procedures decrease the affinity of the peptides for calmodulin. The results are shown in Figure 1. The enhancements by both C28A and C28Y were about one-fifth of that of C28W. The saturation profiles in the presence of peptides C28A and C28Y were different from that of peptide C28W; i.e., the curve was steeper in the case of C28A, indicating a strong cooperativity of Ca^{2+} binding.

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; F12, tryptic fragment of calmodulin extending from acetyl-Ala1 through Lys75; F34, tryptic fragment of calmodulin extending from Asp78 through Lys148.

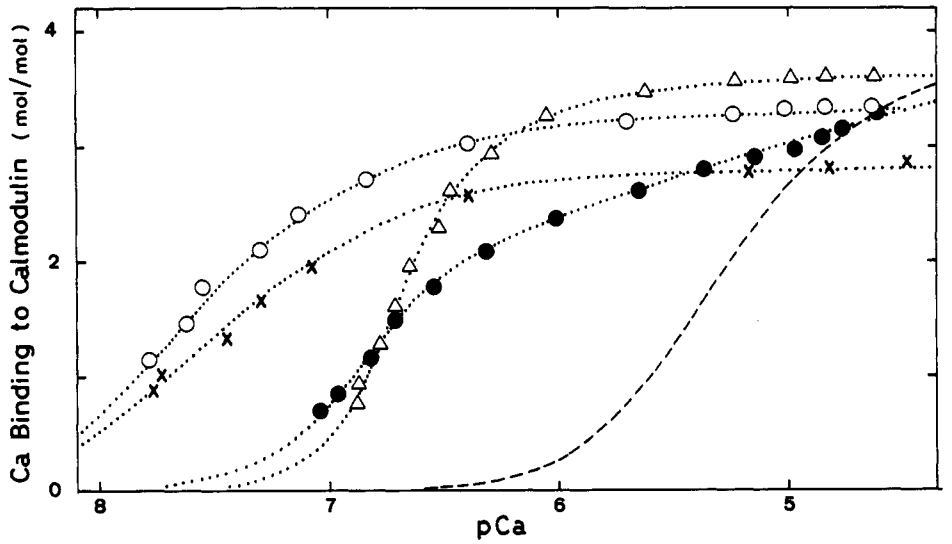

FIGURE 1: Ca^{2+} binding to calmodulin in the presence of the 28-residue peptide C28W and its analogues C28A and C28Y. Ca^{2+} binding was measured by the flow-dialysis method in 0.1 M NaCl, 20 mM MOPS–NaOH (pH 7.0) at 25 °C. The concentration of calmodulin was 16.0 μM. Symbols: ○, +30.4 μM peptide C28W; ×, +60.8 μM peptide C28W; △, +31.5 μM peptide C28A; ●, +30.6 μM peptide C28Y. The dotted lines show the curves which fit best to the Adair equation (eq 1). The dashed line shows the Ca^{2+} binding profile of calmodulin in the absence of peptides (Yazawa et al., 1987).

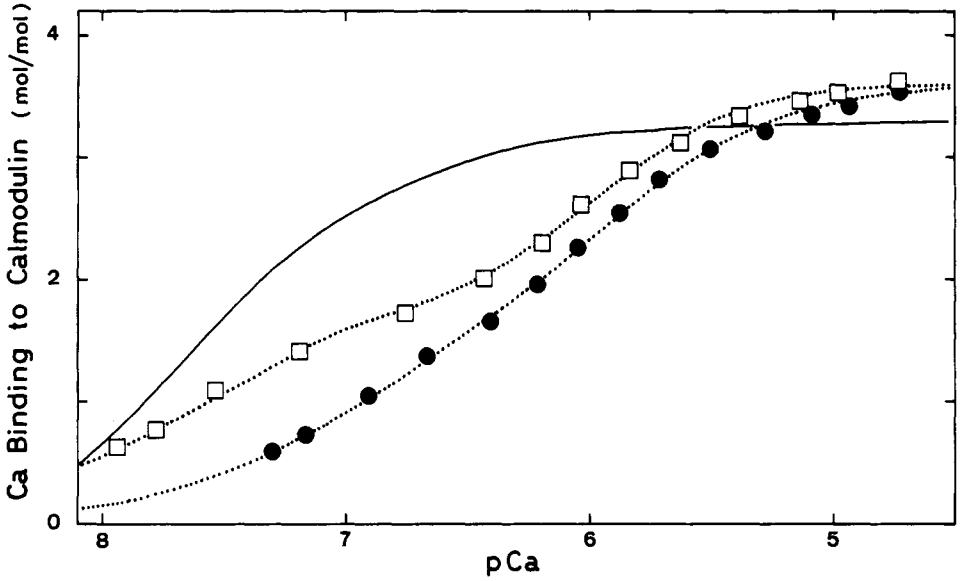

FIGURE 2: Effects of the C-terminal truncation of peptide C28W on Ca^{2+} binding to calmodulin. Ca^{2+} binding was measured under the conditions described in Figure 1 at 16.0 μM calmodulin. Symbols: □, +48.0 μM peptide C20W; ●, +105 μM peptide C15. The solid line shows the best-fit curve for the Ca^{2+} binding of calmodulin in the presence of peptide C28W (transferred from Figure 1). The dotted line shows the best-fit curve for each set of data.

With peptide C28Y, weak Ca^{2+} binding at the low-affinity sites or at the fourth binding site was observed.

Effects of the Deletion of Amino Acid Residues from the C-Terminal Region of C28W. The effects of deleting the C-terminal region of peptide C28W on its effect on the binding of Ca^{2+} by calmodulin are shown in Figure 2. Peptide C20W, which lacks the 8 C-terminal residues, gave a biphasic Ca^{2+} binding profile (Figure 2, □). Apparently, the binding of Ca^{2+} to the high-affinity sites was not affected by the deletion, but the enhancement of the Ca^{2+} affinity of the low-affinity sites was lost.

A larger deletion, removing the 13 C-terminal residues of peptide C28W, decreased the effect on the affinity of the high-affinity Ca^{2+} binding sites (Figure 2, ●) but did not decrease further that on the low-affinity sites. Thus, the domain involved in the interaction of peptide C28W with the high-affinity site of calmodulin could be located between residues 15 and 20 of the peptide. On the other hand, residues 21–28 could be involved in the interaction with the low-affinity site or be related to the interdomain interaction which could be required for the enhancement of the Ca^{2+} affinity of the four sites acting together.

Ca^{2+} Binding to the Tryptic Fragments of Calmodulin. As reported previously (Minowa & Yagi, 1984; Ikura et al., 1984), the half-molecular tryptic fragments (F12 and F34) of calmodulin retained the Ca^{2+} binding properties of the intact protein. F12 (acetyl-Ala1–Lys75) retained the low-affinity binding sites and F34 (Asp78–Lys148) retained the high-affinity binding sites (Figure 3) (Minowa & Yagi, 1984). Upon addition of an approximately equimolar amount of peptide C28W, both the Ca^{2+} affinity of F12 and that of F34 were enhanced (Figure 3). The apparent increase in affinity was 40 times for F34 and 6 times for F12. In the target-free state, the C-domain of calmodulin is assumed to contain the high-

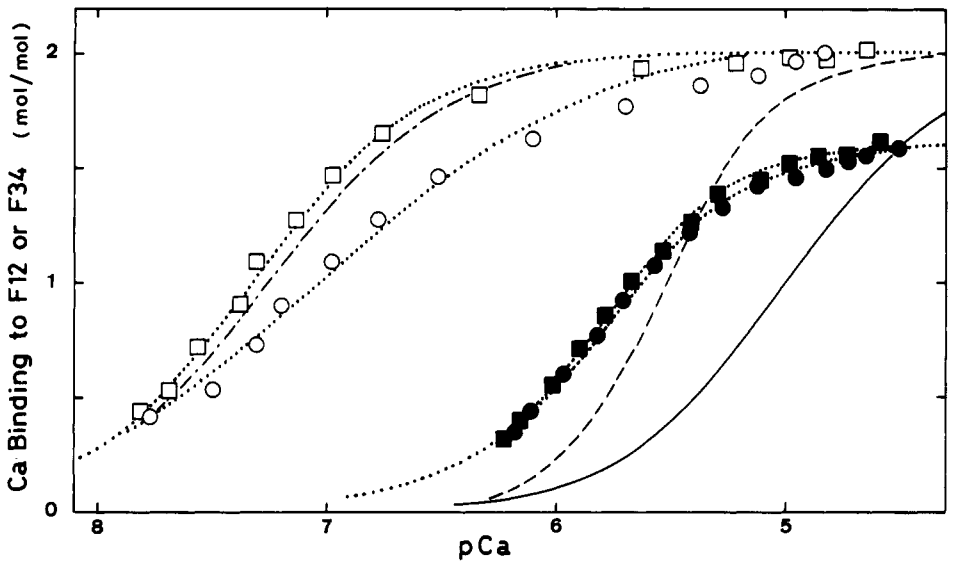

FIGURE 3: Effects of peptide C28W and C20W on Ca^{2+} binding to the two half-molecular fragments of calmodulin, F12 and F34. Ca^{2+} binding was measured under the conditions of Figure 1 at 28.4 μM F12 or 27.1 μM F34 in the presence of 30.4 μM peptide C28W or 35.2 μM peptide C20W. Symbols: ○, F34 + C28W; □, F34 + C20W; ●, F12 + C28W; ■, F12 + C20W. The dotted lines show the best-fit curves. Results of F34 + C28W (○) were corrected assuming that the maximum Ca^{2+} binding of F34 + C28W is attained at pCa 6 (r = 1.65), and the resulting best-fit curve was shown by −·−. The solid and dashed lines are the Ca^{2+} binding curves for F12 and F34, respectively (Minowa & Yagi, 1984).

affinity sites for Ca^{2+} (Minowa & Yagi, 1984; Ikura et al., 1984). It could thus provide the high-affinity binding sites in the calmodulin–C28W complex.

The effect of peptide C20W is also shown in Figure 3: It was very similar to that of peptide C28W for both fragments of calmodulin. This is at variance with the different enhancements of the Ca^{2+} affinity of calmodulin in the presence of these peptides (Figure 2). No difference in the Ca^{2+} binding profiles of F12 was found in the presence of C28W or C20W, and the maximal Ca^{2+} binding (1.6 mol/mol) was found to be lower than 2.0. However, a detailed study of the Ca^{2+} binding by F12 by Minowa and Yagi (1984) had previously yielded a value of 2.0 as the maximal Ca^{2+} binding number: thus, the experimental value of 1.6 mol/mol found in this work may be assumed to correspond to 2.0. The result would rule out the possibility of direct interaction of the 8 C-terminal residues of peptide C28W with the low-affinity sites in the N-domain. These residues could instead be involved in the interdomain interaction. The Ca^{2+} binding to F34 and its cooperativity were apparently stronger with the complex of C20W than with the complex of C28W. In the absence of the peptide, F34 showed the highest degree of cooperativity (Figure 3). Considering the results obtained with calmodulin, peptide C28W could thus decrease the cooperativity in Ca^{2+} binding. However, this could also reflect the solubility effect of C28W as observed in the calmodulin complex. If the latter possibility were true, the increase in Ca^{2+} binding observed beyond pCa 6 could be artifactual and the true Ca^{2+} binding curve of the complex C28W–F34 would be the one at the lower Ca^{2+} concentration (pCa > 6). The dotted broken line in Figure 3 was obtained from the results of the complex F34–C28W, assuming that saturation of F34 occurred at pCa 6: The line is similar to the Ca^{2+} binding curve of the complex F34–C20W. On comparing the three Ca^{2+} binding curves of calmodulin, F34, and F12 in the associated states with peptide C20W (Figures 2 and 3), the C-terminal domain (F34) of calmodulin can be unambiguously assigned as the high-affinity site for Ca^{2+}. The remarkable difference in the enhancement of the Ca^{2+} affinity of the two fragments suggests that the C-domain of calmodulin has higher affinities for peptide C28W and C20W than that of the N-domain.

Ca^{2+} Binding to the Equimolar Mixture of F12 and F34 in the Presence of Peptide C28W or C20W. In the absence of a target protein, the Ca^{2+} binding profile of calmodulin is almost identical to that of the equimolar mixture of F12 and F34 (Minowa & Yagi, 1984). This indicates that no interaction occurs between the N- and C-domains of calmodulin. The dumbbell shape of the molecule in the crystalline state (Babu et al., 1988) conveniently explains this result. The binding of Ca^{2+} to calmodulin and to an equimolar mixture of F12 and F34 was compared in the presence of peptides C28W or C20W (Figure 4). Although the mixture of F12 and F34 is considered here as equimolar, there was in fact a small difference in their concentrations. Therefore, the calculation of the concentration of the mixture was based on the molar concentration of F34. As shown in Figure 4, the resulting Ca^{2+} binding curves were biphasic, irrespective of whether peptide C28W or C20W was used; they were completely different from the corresponding curves of calmodulin (Figure 4). The difference in the complex with peptide C28W is remarkable, since the Ca^{2+} binding curve of the calmodulin complex can be regarded as monophasic. In the presence of peptide C28W the Ca^{2+} binding profile of the equimolar mixture of F12 and F34 was close to the sum (Figure 4A) of the Ca^{2+} binding curves of F12 plus peptide C28W and F34 plus peptide C28W. However, it was completely different from the Ca^{2+} binding curve of the complex calmodulin–peptide C28W (Figure 4A). Apparently, peptide C28W recognizes the difference between the calmodulin molecule and the mixture of the fragments: Calmodulin expressed a monophasic curve and a much stronger Ca^{2+} binding than the fragments. At variance with C28W, the Ca^{2+} binding curve of the equimolar mixture of F12, F34, and peptide C20W was similar to the sum (Figure 4B) of the curves for the complex F34–C20W and target-free F12. The sum (Figure 4B) of the curves for F12–C20W and F34–C20W was also similar to the Ca^{2+} binding curve (Figure 4B) of calmodulin in the presence of a 3-fold molar excess of peptide C20W. In the equimolar mixture of F12, F34, and peptide C20W, the latter may bind to F34 and F12 may be C20W-free. It is likely that in the

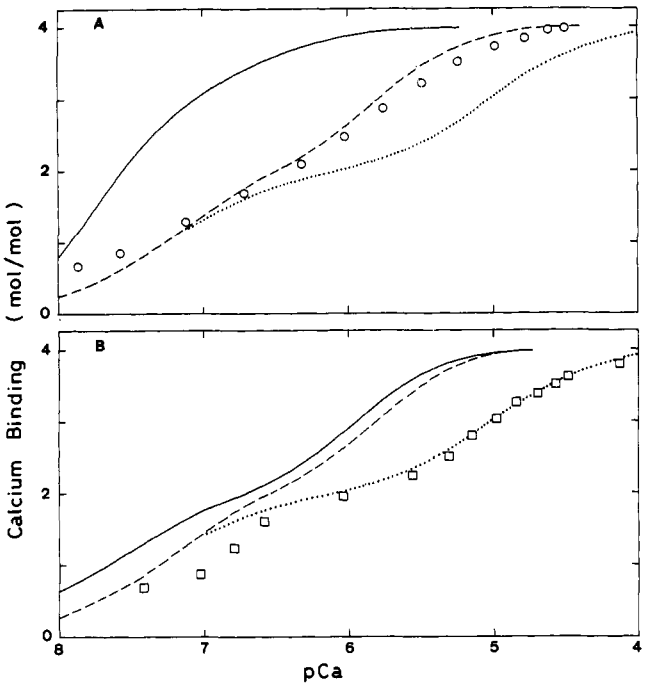

FIGURE 4: Comparison of Ca^{2+} binding to calmodulin and to an equimolar mixture of F12 and F34 in the presence of peptides C28W or C20W. The experimental conditions are the same as in Figure 1, and the Ca^{2+} binding of the equimolar mixture of F12 and F34 was calculated on the basis of the concentration of F34. (A) Results in the presence of peptide C28W: ○, 28.4 μM F12 + 27.1 μM F34 + 30.4 μM peptide C28W. (B) Results in the presence of peptide C20W: □, 31.2 μM F12 + 27.1 μM F34 + 35.2 μM peptide C20W. The Ca^{2+} binding curve of calmodulin in the presence of each peptide is shown by the solid line (the best-fit curves in Figure 2). The dashed line represents the sum of the Ca^{2+} binding curves of F12 and F34 (the best-fit curves in Figure 3), both in the presence of each peptide, and the dotted line is the sum of the Ca^{2+} binding curve of F34 in the presence of each peptide and Ca^{2+} binding curve of F12 without peptide (Figure 3).

Ca^{2+}-saturated state F34 has a higher affinity for C20W than F12. A second molecule of C20W would bind to F12 to produce the curve shown by the dashed line in Figure 4B when more than a 2-fold molar excess of C20W was added. The binding of Ca^{2+} to the two domains of calmodulin is apparently independent in the mixture of calmodulin and peptide C20W, as in the case of target-free calmodulin.

The Ca^{2+}-dependent enhancements of the affinity of the peptides for calmodulin or its fragments (K_d'/K_d) are shown in Table I together with the Ca^{2+} dissociation constants (K_is and K_i's) which were obtained from the best-fit curves in Figures 1 to 3. The affinity of calmodulin for peptides C28W and C20W increased 10^8- and 10^6-fold, respectively, by the binding of Ca^{2+} to calmodulin. Similarly, Ca^{2+} binding to F12 or F34 increased their affinity for the peptides. Since the increase in affinity for F34 was about 100 times higher than for F12, the interaction of the C20W portion of peptide C28W with F34 appears to be stronger than the interaction with F12.

Discussion

The calmodulin binding domain of the erythrocyte Ca^{2+} pump is located in the C-terminal region of the molecule (James et al., 1988; Verma et al., 1988). By using NMR, CD, or fluorescence spectroscopy, it was demonstrated that the synthetic domain, a 28-residue peptide (C28W), had a very high affinity for calmodulin (K_d, less than 1 nM). A C-terminally truncated 20-residue peptide (C20W) had lower calmodulin affinity (K_d = 11 nM) (Vorherr et al., 1990). A further deletion of 5 residues from the C-terminus lowered the affinity of the domain for calmodulin to a K_d of 1.7 μM. A similar trend was detected when the peptides were tested for their ability to inhibit the Ca^{2+} pump activity as calmodulin antagonists (Enyedi et al., 1989).

Table I: Ca^{2+} Binding to Calmodulin or Its Fragments in the Presence of Peptides C28W and C20W[a]

peptide	protein	macroscopic binding constants[a] ($\times 10^6$ M^{-1}) K_1	K_2	K_3	K_4	K_d'/K_d[a]
C28W	calmodulin	32	130	15	8.0	6.2×10^8
C28W	F12	0.56	0.69			45
C28W	F34[b]	37	3.1			1.2×10^3
C28W	F34[c]	28	11			3.2×10^3
C20W	calmodulin	83	25	0.67	1.3	2.2×10^6
C20W	F12	0.46	0.94			50
C20W	F34	29	16			4.8×10^3
	calmodulin[d]	0.35	0.40	0.12	0.048	
	F12[d]	0.14	0.062			
	F34[d]	0.054	1.8			

[a] Each set of macroscopic Ca^{2+} binding constants gives the corresponding best fit curve shown in Figures 1–3. The expected enhancements (K_d'/K_d) of the peptide affinity for calmodulin or fragments were calculated according to eq 2. [b] Values for Figure 3 (○). [c] Values for Figure 3 (–·–). [d] Results of Minowa and Yagi (1984).

The results presented here on the affinity of the calmodulin–peptide complex for Ca^{2+} (Figures 1 and 2) are in line with these results, since they predict that peptide C28W should have 100 times higher affinity for calmodulin than peptide C20W (Table I), assuming an equally low affinity of the two peptides for Ca^{2+}-free calmodulin. The role of Trp8 of the calmodulin binding domain in the affinity of the peptides for calmodulin was consistent with the suggestion of Vorherr et al. (1990), since calmodulin showed weaker Ca^{2+} affinity in the presence of Trp8-substituted analogue peptides (C28A or C28Y) than in the presence of C28W (Figure 1). A similar tendency was confirmed with the 20-residue analogues of C20W (data not shown).

In the presence of target proteins, calmodulin showed much higher affinity for Ca^{2+} than in the target-free state and the Ca^{2+} binding showed higher cooperativity (Olwin & Storm, 1985; Yazawa et al., 1987). A similar tendency was observed in the presence of calmodulin binding peptides such as melittin and mastoparan (Maulet & Cox, 1983; Yazawa et al., 1987). Since the ^1H NMR signal of His108 in the C domain was affected by the Ca^{2+} binding to the N domain in the presence of mastoparan, we have proposed an interdomain interaction which is important in the activation of target enzymes (Yazawa et al., 1987, 1990). In the present works with the calmodulin binding peptides of the plasma membrane Ca^{2+} pump, peptide C28W showed a similar enhancing effect on the Ca^{2+} affinity of calmodulin. However, hardly any increase in cooperativity was detected (Figure 1). The fairly large deviations of the experimental results may be one of the reasons for the failure to confirm the cooperative behavior, since the results with peptide C28A showed a higher cooperativity. Alternatively, the large increase in Ca^{2+} affinity might be sufficient for the regulation of the enzyme by calmodulin.

A deletion of the 8 C-terminal residues from peptide C28W weakened the high-affinity Ca^{2+} binding to calmodulin (Figure 2). The biphasic Ca^{2+} saturation curve may have resulted from the weaker interaction of C20W with the low-affinity site for Ca^{2+} than with the high-affinity site. On the other hand, no evident differences in the Ca^{2+} affinity of the fragment F12, corresponding to the low-affinity Ca^{2+} binding site of calmodulin, were produced by the truncation (Figure 3). Therefore, a direct interaction of the C-terminal 8 residues of peptide C28W with the N-domain may not be necessary

for strong Ca^{2+} affinity of calmodulin induced by the binding of peptide C28W. This region may cooperate with the N-terminal portion of the peptide, which contains Trp8, resulting in the very high affinity of calmodulin for Ca^{2+} in the presence of C28W. Since hydrophobic interactions are assumed to be important in the calmodulin-dependent regulation of target proteins (Tanaka & Hidaka, 1980), two hydrophobic regions in C28W, QILWF9 in the Trp8-containing N-terminal portion and VVNAF25 in the C-terminal portion, could be directly involved in the cooperative process: the C-terminal sequence VVNAF25 could be essential for the high affinity binding, or possibly for the cooperative binding of four Ca^{2+} molecules to calmodulin. The deletion of 5 additional C-terminal residues from the peptide eliminated the strong Ca^{2+} binding to the high-affinity sites, but it did not decrease further that of the low-affinity sites. A neutral hydrophobic sequence (IQTQI19) in this region could be involved in the strong interaction with the C-domain. Since Vorherr et al. (1990) have shown that the first deletion decreases the affinity of the peptide for calmodulin to less than one-tenth, the hydrophobic interaction between calmodulin and the peptides could have become altered, leading to the loss of the enhanced Ca^{2+} binding observed in the complex with peptide C28W. The very large decrease in the affinity for calmodulin induced by the second deletion (Vorherr et al., 1990) could be related to the loss of a site interacting strongly with the C-domain.

The possibility of strong interaction of the 20 N-terminal residues of peptide C28W with the C-domain of calmodulin was supported by the result shown in Figure 3. Quantitative analysis (Table I) indicated that F34 had 100 times higher affinity for the peptides than F12. Therefore, peptide C20W most probably contains a sequence in its C-terminal portion (QTQI19) able to interact with the C-domain of calmodulin, as discussed above. Figure 4 shows that the Ca^{2+} binding curve of an equimolar mixture of F12, F34, and peptide C28W was close to the sum of the curves for the complexes F12–C28W and F34–C28W. On the other hand, the Ca^{2+} binding curve of an equimolar mixture of F12, F34, and peptide C20W was close to the sum of the curves for the complex F34–C20W and target-free F12. These results suggest that a single molecule of peptide C28W could bind both F12 and F34, whereas peptide C20W could bind only to F34 in the equimolar mixture of F12 and F34. Since the Ca^{2+} binding profile of the calmodulin–peptide C28W complex was different from that of the C28W-mediated ternary complex of F12 and F34 (Figure 4), the interdomain interaction through the central helix of calmodulin appears to be essential for the strong binding of four Ca^{2+}. The resulting overall conformation of calmodulin, most probably the bent structure folded at the central helix (Yazawa et al., 1990; Ikura et al., 1991), may be important for the physiological function.

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