Tryptophan Residues in Caldesmon Are Major Determinants for Calmodulin Binding†

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ABSTRACT: Calmodulin has been shown to interact with the COOH-terminal domain of gizzard h-caldesmon at three sites, A (residues 658−666), B (residues 687−695), and B′ (residues 717−725), each of which contains a Trp residue [Zhan *et al*. (1991) *J. Biol. Chem. 266*, 21810−21814; Marston *et al*. (1994) *J. Biol. Chem. 269*, 8134−8139; Mezgueldi *et al*. (1994) *J. Biol. Chem*. *269*, 12824−12832]. To determine the contribution of each of the three Trp residues in the calmodulin−caldesmon interaction, we have mutated the Trp residues to Ala in the COOH-terminal domain of fibroblast caldesmon (CaD39) and studied the effects on calmodulin binding by fluorescence measurements and using immobilized calmodulin. Wild-type CaD39 binds with a K_d of 0.13×10^{-6} M and a stoichiometry of 1 mol of calmodulin per mol of caldesmon. Replacing Trp 659 at site A or Trp 692 at site B to Ala reduces binding by 22- and 31-fold ($K_d = 2.9 \times 10^{-6}$ and 4.0×10^{-6} M), respectively, and destabilizes the CaD39−calmodulin complex by 1.75 and 1.94 kcal mol^{-1}, respectively. Mutation of both Trp 659 and Trp 692 to Ala further reduces binding with a K_d of 6.1×10^{-6} M and destabilizes the complex by 2.17 kcal mol^{-1}. On the other hand, mutation of Trp 722 at site B′ to Ala causes a much smaller decrease in affinity ($K_d = 0.6 \times 10^{-6}$ M) and results in a destabilization energy of 0.87 kcal mol^{-1}. To investigate the relative importance of the amino acid residues near each Trp residue in the caldesmon−calmodulin interaction, deletion mutants were constructed lacking site A, site B, and site A+B. Although deletion of site A decreases binding of CaD39 to calmodulin by 13-fold ($K_d = 1.7 \times 10^{-6}$ M), it results in tighter binding than mutation of Trp 659 to Ala at this site, suggesting that the residues neighboring Trp 659 may contribute negatively to the interaction. Deletion of site B causes a similar reduction in binding ($K_d = 4.1 \times 10^{-6}$ M) as observed for replacing Trp 692 to Ala at this site, indicating that Trp 692 is the major, if not the only, binding determinant at site B. Deletion of both site A and site B drastically reduces binding by 62-fold. Taken together, these results suggest that Trp 659 and Trp 692 are the major determinants in the caldesmon−calmodulin interaction and that Trp 722 in site B′ plays a minor role.

Caldesmon is an actin-binding protein present in both smooth muscle and nonmuscle cells [for reviews, see Sobue and Sellers (1991), Matsumura and Yamashiro (1993), and Marston and Redwood (1991)]. Smooth muscle h-caldesmon has a M_r of 87 000 and contains three structurally and functionally distinct domains: an NH_2-terminal domain (residues 1−250), a middle domain (residues 251−400), and a COOH-terminal domain (residues 401−756). While all three domains have been shown to bind tropomyosin *in vitro* (Redwood & Marston, 1993; Graceffa, 1987; Watson *et al*., 1990), the NH_2-terminal domain also binds myosin (Velaz *et al*., 1990; Ikebe & Reardon, 1988), and the COOH-terminal domain interacts with actin (Bartegi *et al*., 1990; Mezgueldi *et al*., 1994) and the Ca^{2+}-binding proteins calmodulin (Zhan *et al*., 1991; Zhuang *et al*., 1995; Marston *et al*., 1994) and caltropin (Mani *et al*., 1992). Both NH_2- and COOH-terminal domains of smooth muscle caldesmon are conserved in nonmuscle caldesmon with similar primary structures and functions (Hayashi *et al*., 1991; Bryan & Lee, 1991). The h-caldesmon middle domain, which has a high α-helical content based on secondary prediction and circular dichroism measurements (Wang *et al*., 1991a), is absent in the nonmuscle isoform (Ball & Kovala, 1988). Since both smooth muscle and nonmuscle caldesmon have similar functions *in vitro*, it is not clear what functional role is played by the middle domain.

There is strong *in vitro* evidence supporting a regulatory role for caldesmon in smooth muscle contraction and in nonmuscle cell motility. The COOH-terminal domain of caldesmon inhibits actomyosin ATPase by blocking the interaction of actin and myosin and/or inhibiting a kinetic step of the actomyosin ATPase cycle (Marston & Smith, 1985; Ngai & Walsh, 1984; Valez *et al*., 1989; Lash *et al*., 1986; Horiuchi *et al*., 1986). The inhibition can be released by Ca^{2+}−calmodulin (Horiuchi *et al*., 1986; Sobue *et al*., 1985; Ngai & Walsh, 1984). It is generally agreed that there are multiple Ca^{2+}−calmodulin-binding sites at the COOH-terminal domain of caldesmon. However, there is disagreement concerning the location of the sites and the relative contribution of each site in binding to calmodulin and in the release of caldesmon inhibition of actomyosin ATPase (Wang *et al*., 1996; Marston *et al*., 1994; Zhuang *et al*., 1995; Huber *et al*., 1996).

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It has been proposed that Ca^{2+}−calmodulin interacts with three sites in chicken gizzard caldesmon: M658-W-E-K-G-N-V-F-S666 (site A), S687-R-I-N-E-W-L-T-K695 (site B), and G717-K-R-N-L-W-E-K-Q725 (site B′) (Zhan *et al*., 1991; Marston *et al*., 1994; Mezgueldi *et al*., 1994). Each of the three sites contains a Trp residue, which is believed to be involved in calmodulin binding as has been shown by fluorescence measurements (Shirinsky *et al*., 1988). Recent NMR data by Huber *et al*. (1996) showed the distinct Ca^{2+}−calmodulin interaction with Trp residues 692 and 722 at sites B and B′, respectively. Another report by Wang *et al*. (1996) has shown that there are three calmodulin-binding determinants localized within residues 628−717, which encompasses only sites A and B. However, deletion of residues 718−756, containing site B′, had no effect on calmodulin binding. The apparent discrepancy could be due to the use of small caldesmon fragments of various lengths, which likely have conformations quite different from that of the intact protein or the COOH-terminal domain.

The aim of the present study is to determine the contribution of each Trp residue at sites A, B, and B′ in the caldesmon−calmodulin interaction. In order to minimize disruptions to the conformation of the caldesmon COOH-terminal domain, we have mutated the Trp residue in each site to Ala in the COOH-terminal domain of human fibroblast caldesmon (CaD39) and studied the effects on calmodulin binding. We present here fluorescence and sedimentation binding data showing that Trp 659 and Trp 692, in sites A and B, respectively, are important in Ca^{2+}−calmodulin binding whereas Trp 722 in site B′ plays a minor role. To determine the contribution of the amino acid residues neighboring the Trp residues at each site to calmodulin binding, we have constructed deletion mutants lacking sites A, B, and A+B in CaD39. Binding data using the deletion mutants suggest that amino acids near Trp 692 at site B contribute little, if at all, to calmodulin binding, whereas amino acids surrounding Trp 659 may contribute negatively to binding.

MATERIALS AND METHODS

Protein Preparations. Smooth muscle caldesmon was prepared from chicken gizzard as described by Bretscher (1984). Recombinant COOH-terminal fragment of human fibroblast caldesmon (CaD39) and all mutants were expressed in *Escherichia coli* and purified as described by Novy *et al*. (1993), with the following modifications: the ammonium sulfate precipitation was performed from 0 to 55%, and the pooled samples from the MonoS column were dialyzed against ammonium bicarbonate and lyophilized. Bovine brain calmodulin, which is identical in sequence to chicken gizzard caldesmon, was purified according to the method of Gopalakrishna and Anderson (1982).

All protein concentrations, except for calmodulin, were determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as a standard. Bovine brain calmodulin concentration was determined using the absorption coefficient $A^{1\%}$ at 276 nm = 2.0 (Wolff *et al*., 1977).

In Vitro Site-Directed Mutagenesis. The point mutants replacing Trp with Ala were constructed by the method of Deng and Nickoloff (1992) using the "Transformer site-directed mutagenesis kit, 2nd version" (Clonetech), in the clone pETCaD39, a gift from Dr. J. J.-C. Lin, University of Iowa. The mutagenesis was performed in the pET-3d vector. The primers 5′CATTCCCTTTCTC(CGC)CATACTCT-TGATG3′, 5′GGGTTTTAGTTAG(CGC)TTCATTGAT-GCGGC3′, and 5′CAGATTGCTTTTC(CGC)GAGGTTC-CGCTTGC3′ were used to generate the mutants W659A (W461A), W692A (W494A), and W722A (W524A) and the double mutant W659,692A (W461,494A). The numbering refers to the chicken gizzard sequence (Bryan & Lee, 1991) while the numbering in parentheses refers to the human fibroblast caldesmon sequence (Novy *et al*., 1991). For the sake of simplicity, the chicken gizzard sequence will be used as a reference sequence. Each primer sequence represents the antisense sequence where the mismatched bases are underlined and the sequences that give rise to Ala codons are shown in parentheses. This method takes advantage of a second primer (selection primer, 5′CAGCTTATCATCTA-GACGCGTTAATGCGGTAG3′), which anneals to the same strand of DNA as the mutagenic primer and eliminates the unique *Cla*I and *Hin*dIII sites from the pET-3d backbone of the parental plasmid. Cleavage with these two selection enzymes linearizes the parental plasmid, allowing for the recovery of mutants with very little background of wild-type plasmids.

The deletion mutants removing the calmodulin-binding sites A, B, and A+B were constructed by PCR mutagenesis using the following mutagenic primer pairs: A: 5′TCAA-GAGT/TCCCCCACTGCAGCAG3′, 5′GTGGGGGA/ACTC-TTGATGTTGCGTACA3′; B: 5′GGGTTTCT/ACCCCA-GATGGAAACAAG3′, 5′TCTGGGGT/AGAAACCCCTAC-CTTCAAG3′. The backslashes denote deletion boundaries. The deletion mutants are missing the putative calmodulin-binding sites A and B corresponding to the chicken gizzard h-caldesmon sequences (with the corresponding human fibroblast caldesmon sequences in parentheses): Δ658−666 (Δ460−468) and Δ687−695 (Δ489−497). A double mutant missing both sites A and B, Δ658−666,687−695 (Δ460−468,489−497), was also constructed.

DNA sequence analyses were performed to confirm the identity of the Trp-to-Ala and deletion mutations.

Fluorescence Studies. The binding of calmodulin to CaD39, W659A, W692A, W659,692A, or W722A was monitored by tryptophan fluorescence on a Perkin-Elmer LS-50 spectrometer at 25 °C. A stock solution of calmodulin (0−8 μM final concentration) was titrated into a 1 μM solution of caldesmon fragment such that the total volume change was less than 15%; corrections to the intensity measurements were made for dilution during the titration. The buffer was 20 mM Tris-HCl, pH 7.2, 0.5 mM $CaCl_2$, 100 mM NaCl, 1 mM DTT, and 0.1 mM NaN_3. The excitation wavelength was at 295 nm, and the excitation slit width was 5 nm. Intensity measurements were made with a 290 nm filter at 330 nm, with a slit width of 10 nm.

Binding curves were fitted to the following equation using a nonlinear regression routine:

$$\Delta I/I_o = (\Delta I_{max}/I_o)[A - (A^2 - 4nP_TL_T)^{1/2}]/2nP_T$$

where ΔI = the change in fluorescence intensity in the caldesmon fragment induced by calmodulin, I_o = the fluorescence of the caldesmon fragment in the absence of calmodulin, ΔI_{max} = the maximal change in caldesmon fragment fluorescence saturated with calmodulin, $A = nP_T + L_T + K_d$, n = the number of moles of calmodulin-binding sites per mole of caldesmon fragment, P_T = the total

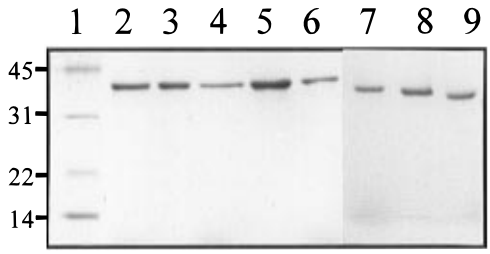

FIGURE 1: Purified mutants of recombinant COOH-caldesmon. SDS−PAGE followed by Coomassie Blue staining of approximately 1 μg of CaD39 or its mutants. The numbers in the left-hand column indicate the molecular mass in kilodaltons. Lane 1, low molecular mass markers (from top to bottom: ovalbumin, 45 000 Da; carbonic anhydrase, 31 000 Da; soybean trypsin inhibitor, 22 000 Da; lysozyme, 14 000 Da); lane 2, CaD39; lane 3, W659A; lane 4, W692A; lane 5, W659,692A; lane 6, W722A; lane 7, ΔA; lane 8, ΔB; lane 9, ΔAB.

caldesmon fragment concentration, L_T = the total calmodulin concentration, and K_d = the dissociation constant.

Binding of CaD39 and Mutants to Immobilized Calmodulin. Calmodulin was coupled to Pharmacia CNBr−Sepharose 4B beads according to the manufacturer's instructions. CaD39 or its mutants (3 μM) were incubated with calmodulin−Sepharose (30 μM) for 30 min in binding buffer (20 mM Tris-HCl, pH 7.2, 0.5 mM $CaCl_2$, 100 mM NaCl, 1 mM DTT, and 0.1 mM NaN_3) at room temperature. The beads were washed twice with binding buffer, and bound caldesmon was eluted twice with 20 mM Tris-HCl, pH 7.2, 10 mM EGTA, 100 mM NaCl, 1 mM DTT, and 0.1 mM NaN_3. The samples were centrifuged at 12000*g* for 30 s between each manipulation. Supernatants from the washes and elutions were subjected to SDS−PAGE, after which the gel was stained with Coomassie Blue and the amount of bound and free protein was determined by scanning densitometry. The binding of BSA and the binding of CaD39 to calmodulin−Sepharose in the presence of 10 mM EGTA were used as controls.

RESULTS

Fragments Corresponding to the COOH-Terminal Domain of Caldesmon Used in This Study. For the present studies, we have chosen to use the recombinant fragment CaD39 which corresponds to the entire COOH-terminal domain of human fibroblast caldesmon, residues 244−538 (residues 443−736 in chicken gizzard β caldesmon). The sequences in CaD39 are highly conserved in the corresponding region in chicken gizzard h-caldesmon; moreover, the amino acid sequences of the three putative calmodulin-binding sites are identical in the two proteins with the exception of a single replacement of Gly 717 in h-caldesmon at site B′ by a Ser in the fibroblast isoform (Bryan & Lee, 1991; Novy *et al*., 1991). This highly conserved domain has been shown to exist as a distinct structural fragment which retains all the known functions inherent in the COOH-terminal domain of the intact protein. For example, it binds actin, tropomyosin, and calmodulin, and it inhibits actin activated myosin ATPase activity (Novy *et al*., 1993; Tsuruda *et al*., 1995). Trp/Ala mutants, W659A, W692A, W659,692A, and W722A, and deletion mutants of CaD39 lacking the putative calmodulin-binding sites A, B, and A+B (ΔA, ΔB, and ΔAB, respectively) were expressed in *E. coli* and purified as described under Materials and Methods. The purified mutants were examined by SDS/PAGE as shown in Figure 1, and a schematic representation of the deletion and Trp/Ala mutants is shown in Figure 2.

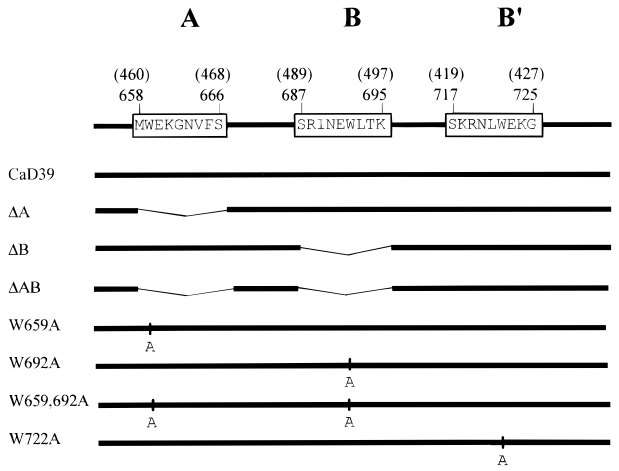

FIGURE 2: Schematic diagram of CaD39 and mutants. CaD39 and its mutants were generated in the *E. coli* expression system. The numbers represent the chicken gizzard sequence; numbers in parentheses represent the human fibroblast caldesmon sequence. Putative calmodulin-binding sites are boxed and show the amino acid residues of human fibroblast caldesmon. The positions of the Trp-to-Ala mutations are indicated with the letter A.

Table 1: Binding Parameters of the CaD39−Calmodulin Interaction[a]

caldesmon fragment	K_d (μM)	$\Delta I_{max}/I_o$ at 330 nm
CaD39	0.13 ± 0.01	0.77 ± 0.03
W659A	2.9 ± 0.5	0.86 ± 0.04
W692A	4.0 ± 0.5	0.97 ± 0.04
W659,692A	6.0 ± 1.0	0.40 ± 0.10
W722A	0.60 ± 0.01	0.96 ± 0.02
ΔA	1.7 ± 0.2	0.62
ΔB	4.1 ± 0.1	0.77
ΔAB	8.9 ± 0.4	0.50

[a] Binding data were obtained from 2 to 5 different preparations of CaD39 and mutants, and at least 2 binding curves were constructed using each protein preparation.

Binding of Trp/Ala Mutants of CaD39 to Ca^{2+}−Calmodulin. In the absence of calmodulin, the emission spectra of CaD39 and all the Trp/Ala mutants are similar in shape to that of the chicken gizzard caldesmon and exhibit a maximum fluorescence intensity at 348 nm. The relative fluorescence intensities (I_o at 348 nm) of 1 μM of CaD39, W659A, W692A, W659,692A, and W722A are about 100: 64:62:30:65, indicating that each Trp residue contributes equally to the total fluorescence of CaD39, that is, about 32%, 35%, and 30% for Trp 659, Trp 692, and Trp 722, respectively.

Calmodulin itself does not contain Trp residues, but it increased the fluorescence intensity of all three Trp residues in CaD39 by 77%. Replacement of any one of the three Trp residues by Ala results in an augmented calmodulin-induced enhancement of fluorescence in the remaining two Trp residues as shown by the values of $\Delta I_{max}/I_o$ at 330 nm (Table 1). These data suggest that the binding of calmodulin to CaD39 affects each Trp residue to a differing degree and the effect on each Trp residue is not independent of the others.

Calmodulin binding also caused a blue shift in the emission maximum from 348 to 337 nm, indicating that calmodulin either directly or indirectly shields the Trp residues from a polar environment. Our observation is in agreement with

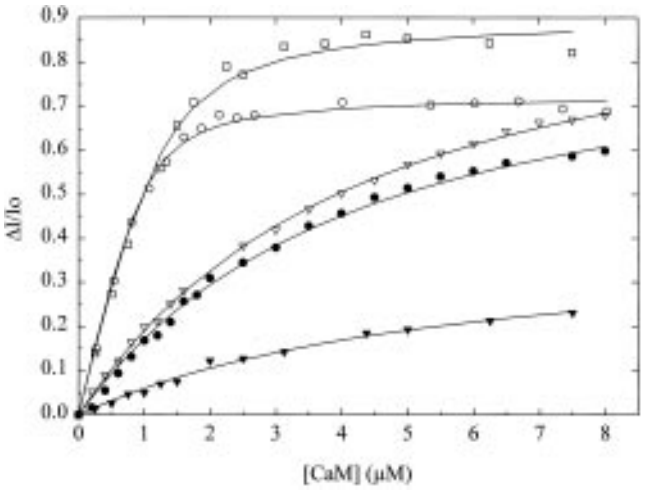

FIGURE 3: Binding of CaD39 and Trp/Ala mutants to calmodulin. The binding of calmodulin (0−8 μM) to CaD39 (open circles), W659A (filled circles), W692A (open triangles), W659,692A (filled triangles), or W722A (open squares) was monitored by tryptophan fluorescence. The lines indicate the fitted curves. The buffer was 20 mM Tris-HCl, pH 7.2, 0.5 mM $CaCl_2$, 100 mM NaCl, 1 mM DTT, and 0.1 mM NaN_3. Fluorescence measurement was carried out at 25 °C, with the excitation wavelength at 295 nm and excitation slit at 5 nm. Measurements were made with a 290 nm filter at 348 nm with a 10 nm emission slit.

the original report by Shirinsky *et al*. (1988), who observed a shift of the emission maximum from 351 to 339 nm in gizzard h-caldesmon in the presence of calmodulin. Calmodulin induced changes in the intensity, and a shift in the maximum wavelength of the emission of CaD39 was abolished when EGTA was present, indicating a specific Ca^{2+}-dependent interaction (data not shown).

To determine the contribution of each Trp residue in sites A, B, and B′ in the calmodulin−caldesmon interaction, binding curves (Figure 3) were constructed and fitted to the equation described under Materials and Methods. CaD39 binds calmodulin with a K_d of 0.13×10^{-6} M, and $n = 1.1$ (see Table 1). This is in agreement with previous reports (Huber *et al*., 1996; Zhuang *et al*., 1995) that although the caldesmon and calmodulin interaction involves multiple contact sites, 1 mol of caldesmon binds 1 mol of calmodulin. Replacing Trp 659 or Trp 692 with Ala at site A or B reduces binding by 22- and 31-fold ($K_d = 2.9 \times 10^{-6}$ and 4.0×10^{-6} M), respectively. Mutating both Trp residues at 659 and 692 to Ala decreases binding further, resulting in a reduction in binding of almost 50-fold ($K_d = 6.1 \times 10^{-6}$ M). Replacing Trp 722 with Ala at site B′ has a relatively small effect on the binding of CaD39 to calmodulin, resulting in a less than 5-fold reduction in binding ($K_d = 0.60 \times 10^{-6}$ M). These data indicate that while both Trp 659 and Trp 692 are major determinants in the caldesmon−calmodulin interaction, Trp 722 plays a minor role.

To measure the interaction energy of the Trp residues in caldesmon with calmodulin, we calculate the apparent binding energy as defined by Fersht (1988), ΔG_{app}, which compares the binding energy of a wild-type protein with that of a mutant in which the interacting side chain has been truncated and replaced by a hydrogen atom. In our case, the indole ring at the β-carbon of each Trp residue has been deleted and replaced by a hydrogen atom to generate an Ala residue. ΔG_{app} measures the difference between the binding energies of the CaD39−calmodulin interaction and the CaD39 mutant−calmodulin interaction. Thus, $\Delta G_{app} = RT \ln (K_d/K_d')$, where K_d and K_d' are the dissociation constants of the calmodulin−CaD39 and calmodulin−mutant complexes, respectively. Replacing the indole ring of Trp 659 or Trp 692 with a hydrogen atom causes a similar destabilization of the calmodulin−CaD39 complex, that is, by 1.75 and 1.94 kcal mol^{-1}, respectively (Table 2). Mutation of both Trp 659 and Trp 692 causes a further destabilization, $\Delta G_{app} = -2.17$ kcal mol^{-1}. A similar mutation of Trp 722, however, has a significantly less destabilizing effect as reflected in the ΔG_{app} value of -0.87 kcal mol^{-1}.

Table 2: Binding Energy of the Trp/Ala Mutant−Calmodulin Interaction

caldesmon fragment	ΔG_{app} (kcal mol^{-1})[a]
W659A	−1.75
W692A	−1.94
W659,692A	−2.17
W722A	−0.87

[a] $\Delta G_{app} = RT \ln (K_d/K_d')$, where K_d and K_d' are dissociation constants of the CaD39−calmodulin and Trp/Ala mutant−calmodulin complexes, respectively.

To confirm the binding data based on fluorescence measurements, we have carried out binding experiments using calmodulin immobilized on Sepharose beads, which allowed us to measure directly the amount of caldesmon fragment bound. As shown in Figure 4A, the double mutant, W659,692A, has the lowest affinity for calmodulin while W659A and W692A have a similar decrease in their affinity for calmodulin. Replacing Trp 722 with Ala shows little effect on the binding of CaD39 to calmodulin. Negligible amounts of bound protein were recovered when EGTA was included in the binding assay or BSA was used as the ligand, indicating specific binding (data not shown). These results therefore confirm the fluorescence data that Trp 659 and Trp 692 are shown to be equally important in the binding of caldesmon to calmodulin, whereas Trp 722 is less so. It should be pointed out that the margins of error in the affinity binding assays are higher than those in fluorescence measurements especially for the weak-binding mutants. This is likely due to the uncertainty of the accessibility of the immobilized calmodulin to CaD39 and mutants, and the cumulative error involved in the subsequent steps of analyses of bound protein. For this reason, no attempt was made to calculate binding parameters based on the affinity binding data. However, affinity data provide a semiquantitative analysis of direct binding and help in confirming the fluorescence results.

Interaction of Calmodulin with Deletion Mutants of CaD39 Lacking Calmodulin-Binding Sites A and B. To investigate the involvement in calmodulin binding of amino acid residues other than Trp in sites A and B, we have generated deletion mutants lacking site A (ΔA, residues 658−666, Met-Trp-Glu-Lys-Gly-Asn-Val-Phe-Ser), site B (ΔB, residues 687−695, Ser-Arg-Ile-Asn-Glu-Trp-Leu-Thr-Lys), and both sites A and B (ΔAB, residues 658−666 and 687−695). As shown in Table 1 and Figure 5, calmodulin induces an increase in $\Delta I_{max}/I_o$ at 330 nm by about 62% and 77% in ΔA and ΔB, respectively, and causes a shift in the emission maximum from 348 to 339 nm. Deletion of site A reduces the binding of CaD39 to calmodulin by 13-fold with a K_d of 1.7×10^{-6} M; interestingly, ΔA has a higher affinity for calmodulin than the W659A mutant which has a K_d of 2.9×10^{-6} M. It appears that the amino acid residues neighboring Trp 659 at site A contribute negatively to calmodulin binding. On the

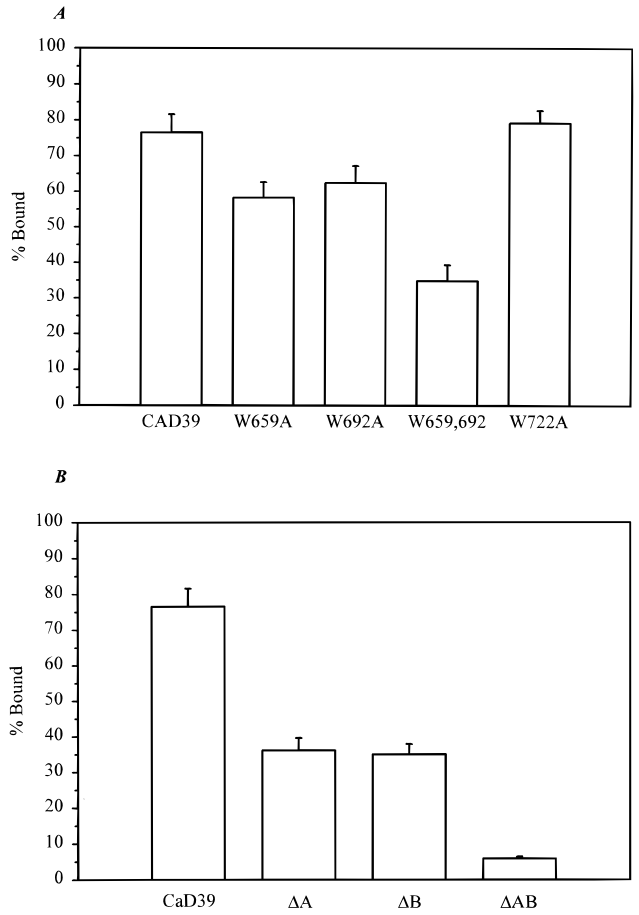

FIGURE 4: Binding of CaD39, Trp/Ala, and deletion mutants to calmodulin immobilized on Sepharose beads. Binding of calmodulin (30 μM) to CaD39 fragments (3 μM) was performed as outlined under Materials and Methods. Buffer conditions were identical to those used in Figure 3. % Bound represents the amount of caldesmon bound over the total amount of caldesmon added. (*A*) CaD39 and Trp/Ala mutants; (*B*) CaD39 and deletion mutants.

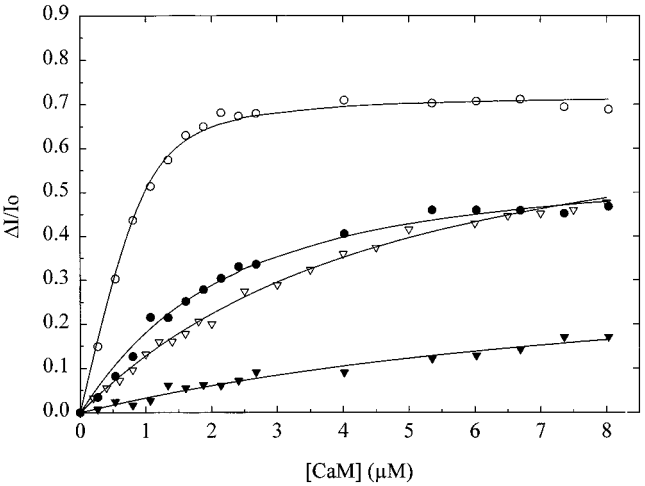

FIGURE 5: Binding of CaD39 and deletion mutants to calmodulin. The binding of calmodulin (0−8 μM) to CaD39 (open circles), ΔA (filled circles), ΔB (open triangles), or ΔAB (filled triangles) was monitored by tryptophan fluorescence. The lines indicate the fitted curves. Conditions for the buffer and fluorescence measurements are identical to those in Figure 3.

other hand, deletion of site B causes an almost identical degree of reduction in binding as observed when Trp 692 alone is replaced by an Ala (K_d for ΔB and W692A are both about 4×10^{-6} M). This result suggests that Trp 692 in site B is the predominant binding determinant with little or no contribution from the neighboring amino acid residues. Deletion of both sites A and B reduces binding by almost 70-fold, and calmodulin induces changes in $\Delta I_{max}/I_o$ of the remaining Trp 722 by about 50%, but causes a smaller shift in the emission maximum from 348 to 343 nm.

The fluorescence data were again confirmed by direct binding analyses using calmodulin immobilized on Sepharose beads. As shown in Figure 4B, deletion of both sites A and B essentially abolishes the binding of CaD39 to calmodulin under these binding conditions. Deletion of either site A or B reduces the amount of bound caldesmon fragments by about half.

DISCUSSION

The general approach employed by many previous studies to localize the calmodulin-binding sites on caldesmon was to identify calmodulin-binding fragments of various sizes representing the different regions of the COOH-terminal domain of caldesmon. Thus, it was first demonstrated that the major calmodulin-binding sites reside in the chymotryptic fragments corresponding to the COOH-terminal region of caldesmon (Wang *et al*., 1991b). Synthetic peptides corresponding to residues 658−666 (Zhan *et al*., 1991) and 675−695 (Mezgueldi *et al*., 1994) in chicken gizzard h-caldesmon were later shown to bind calmodulin in a Ca^{2+}-dependent manner. Using recombinant fragments, Marston *et al*. (1994) proposed that Ca^{2+}−calmodulin interacts with three distinct caldesmon sites: residues 658−666 (site A), residues 687−695 (site B), and residues 717−726 (site B′). This strategy has been successful in providing us with crucial information about the calmodulin-binding sites, especially since the detailed three-dimensional structure of caldesmon is unknown. However, accurate interpretation of binding data using this approach relies on the assumption that the fragments retain their "native" conformations. Although such an assumption is valid for most large fragments representing functional and structural domains, smaller fragments, depending on the location of the ends, may generate false positive or negative binding results. Recently, Wang *et al*. (1996) have attempted to address this problem by producing caldesmon mutants with truncated COOH-terminal sequences of various lengths. Their studies showed that removing 38 amino acid residues containing the putative calmodulin-binding site B′ from the COOH-terminus did not affect calmodulin binding and thus did not demonstrate site B′ as a calmodulin-binding site as previously reported by Marston *et al*. (1994).

In order to minimize the disruption to the overall conformation of the COOH-terminal domain and to investigate the involvement of the Trp residues in calmodulin binding, we have replaced each Trp residue in sites A, B, and B′ with Ala and studied their binding with calmodulin. We have shown that Trp 659 and Trp 692 in sites A and B are the major binding determinants since replacement of Trp 659 or Trp 692 with Ala reduces the binding of CaD39 to calmodulin by about 20- and 30-fold, and destabilizes the CaD39−calmodulin complex by 1.75 and 1.94 kcal mol^{-1}, respectively. Replacing Trp 722 in site B′ with Ala has a much smaller effect on the interaction between caldesmon and calmodulin, causing about a 4-fold reduction in binding and a destabilization energy of 0.87 kcal mol^{-1}, although a slight increase in $\Delta I_{max}/I_o$ (Table 1 and Figure 3) is observed.

These data indicate that although all three Trp residues at sites A, B, and B′ contribute to the calmodulin−caldesmon interaction, Trp 659 and Trp 692 play a much greater role than Trp 722. This suggests a certain flexibility in the multiple-site interaction between caldesmon and calmodulin, such that sites A and B act as primary anchoring sites. Site B′, in contrast, may act as a secondary site whose binding is easily reversible depending on the environment surrounding the thin filament. The weak interaction between site B′ and calmodulin may also explain why some have observed an interaction between calmodulin and caldesmon fragments containing site B′ (Huber *et al*., 1996; Marston *et al*., 1994), while others (Wang *et al*., 1996) did not detect significant changes in binding when the region containing site B′ (residues 718−756) was truncated from caldesmon.

Deletion of the nine residues corresponding to site A (residues 658−666) from CaD39 reduces its binding to calmodulin as expected; however, calmodulin has a higher affinity for this deletion mutant than for W659A where Trp 659 is replaced by an Ala. This observation suggests that the amino acid residues near Trp 659 may have a negative effect on calmodulin binding. Since site A contains the sequence Met-Trp659-Glu-Lys-Gly-Asn-Val-Phe-Ser, it is conceivable that the charged Glu and Lys residues next to Trp 659 may interfere with hydrophobic interactions involving this Trp residue. On the other hand, amino acids near Trp 692 in site B contribute little to the caldesmon−calmodulin interaction since deletion of site B reduces binding to a similar extent as replacing Trp 692 by an Ala.

It has been shown that calmodulin binds strongly to basic, amphiphilic, and α-helical motifs of about 20 residues. These are frequently found in calmodulin-binding sites of proteins that have a high affinity for calmodulin (K_d ranges from 10^{-9} to 10^{-8} M) (O'Neil & DeGrado, 1990). It is apparent that the hydrophobic and electrostatic interactions both contribute to the binding of these calmodulin-binding motifs. For caldesmon, the amino acid sequences in and neighboring sites A, B, and B′ bear little resemblance to the high-affinity basic, amphiphilic α-helical motif and probably represent a low-affinity binding motif belonging to calmodulin-binding proteins with a K_d in the 10^{-6} M range. While a typical calmodulin-binding domain is believed to consist of a continuous sequence, some calmodulin-binding proteins have multiple binding sites and bind more than 1 mol equiv of calmodulin (Goold & Baines, 1994; Menegazzi *et al*., 1994). Caldesmon is unusual in that it has multiple calmodulin-binding sites but binds 1 mol equiv of calmodulin. Our finding that all three sites in caldesmon are involved to differing degrees with calmodulin binding is consistent with the model suggested by Marston *et al*. (1994) and Huber *et al*. (1996), who proposed a multisite interaction between caldesmon and both NH_2- and COOH-terminal lobes of calmodulin.

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