Mutational Effects on the Cooperativity of Ca^{2+} Binding in Calmodulin†

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Received March 4, 1993; Revised Manuscript Received April 30, 1993

ABSTRACT: The importance of the aspartate ligand in the +Y Ca^{2+} coordinating position of two EF-hands of calmodulin has been investigated. Synthetic calmodulin genes were used to produce engineered proteins with the wild-type bovine sequence as well as with aspartate 58 in Ca^{2+}-binding site II and/or aspartate 95 in site III changed to asparagine. The macroscopic Ca^{2+}-binding constants of the intact calmodulins and of tryptic fragments comprising the N- and C-terminal domains were determined from titrations with Ca^{2+} in the presence of 5,5′-Br_2BAPTA. Substitution of aspartate by asparagine in Ca^{2+}-binding site II led to a slight increase in the total free energy change on Ca^{2+} binding, and the cooperativity of Ca^{2+} binding to the N-terminal sites was substantially increased. The change from aspartate to asparagine in site III decreased the Ca^{2+} affinity and also appeared to decrease the positive cooperativity between the sites in the C-terminal domain. Thus, identical mutations in sites II and III were found to result in opposite effects. The data imply that involvement of liganding side chains in interactions other than direct calcium attraction and calcium coordination is of considerable importance for the Ca^{2+}-binding process, particularly for the cooperativity.

Calmodulin is a regulatory, intracellular Ca^{2+}-binding protein that is present in all eukaryotic cells from yeast to higher mammals and is highly conserved throughout evolution. Ca^{2+} binding to calmodulin induces conformational changes, thereby allowing calmodulin to regulate numerous enzymes.

The crystal structure of calcium-loaded calmodulin (Babu et al., 1985, 1988; Kretsinger & Weissman, 1986) resembles a dumbbell, with a central helix connecting two globular domains. Each domain contains two EF-hand (Kretsinger & Nockolds, 1973) calcium-binding sites. The central helix is more flexible in solution than in crystals due to disruptions near its midpoint (Persechini & Kretsinger, 1988; Barbato et al., 1992). The flexibility of the central helix allows the two globular domains to come together when binding to peptides that comprise calmodulin binding-domains of target enzymes. This leads to a globular conformation of the calmodulin–peptide complex in solution (Ikura et al., 1992) and in crystals (Meador et al., 1992).

In the presence of target peptides, Ca^{2+} binding appears to be cooperative among all four binding sites (Yazawa et al., 1987; Ikura et al., 1989). However, in free calmodulin the binding of two Ca^{2+} ions to each globular domain is positively cooperative, but there is no interdomain cooperativity (Linse et al., 1991a). Ca^{2+}-binding sites I and II, which are in the N-terminal domain, have lower Ca^{2+} affinity than sites III and IV, which reside in the C-terminal domain (Thulin et al., 1984; Wang et al., 1984; Martin et al., 1985).

In an EF-hand the Ca^{2+} ion is coordinated by seven oxygen ligands in a pentagonal bipyramidal configuration. In general, five of these oxygens are provided by side chains, one by a backbone carbonyl, and one by a water molecule. The calcium ligands provided by the protein are all within a 12-residue segment termed the loop, although the ends are actually within the helices. The backbone and side chains in this loop are involved in an extensive hydrogen-bond network that is highly conserved among the EF-hand Ca^{2+}-binding proteins (Strynadka & James; 1989). The ligand in position +Y is most frequently an aspartate or asparagine residue that contributes one side-chain oxygen to calcium coordination. All four loops in calmodulin have Asp in this position, which is the third residue in the loop (Figure 1). The crystal conformations of loops II and III (Babu et al., 1988) are shown in stereo in Figure 2. As shown in this figure, the second carboxylate oxygen of Asp in the +Y position is not directly coordinating the calcium ion. Instead, it is hydrogen bonded to the calcium-coordinating water in position –X. The same water molecule is also hydrogen bonded to the side chain of amino acid 9 in the loop, which is an aspartate in loop II and a serine in loop III. An antiparallel β-sheet between the two loops in each globular domain involve positions 7–9. In addition, the backbone NH of the ninth residue forms a hydrogen bond to the carboxylate of the residue in position –Z, which is

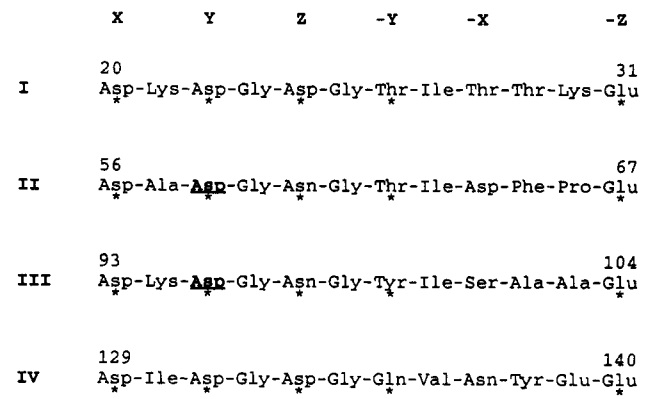

FIGURE 1: Amino acid sequences of the four calcium-binding sites in calmodulin. Residues forming the Ca^{2+} coordination sphere are denoted X, Y, Z, –Y, and –Z and are indicated by stars. The numbers represent the positions in the amino acid sequence of calmodulin. The calcium coordination sphere is completed by a water molecule in position –X.

† This work was supported by the Swedish National Board of Technical Development. T.G. was the recipient of a special researcher position from the Swedish Natural Science Research Council.

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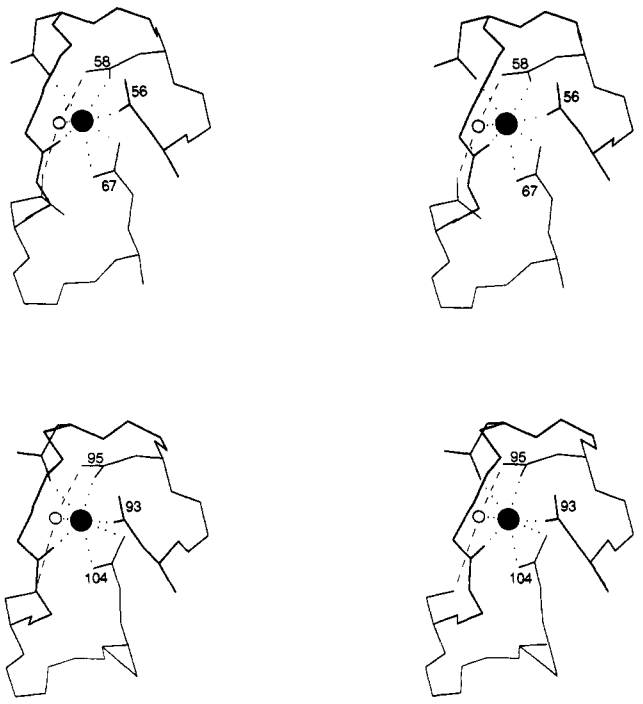

FIGURE 2: Stereoscopic views of 12-residue loops in calmodulin: top, loop II; bottom, loop III. The Ca^{2+} ions (●) are connected to their coordinating ligands by dotted lines. Hydrogen bonds involving the water molecule (O) in position –X are shown with dashed lines. The terminal residues of the loops and aspartates 58 and 95 are indicated. Both loops are oriented such that the β-strand is to the left in the figure.

invariantly a glutamate that contributes both carboxylate oxygens to Ca^{2+} coordination.

Many mutational studies of EF-hand proteins and synthetic peptide analogues focus on calcium ligands. The role of the glutamate in position –Z has been the subject of several investigations [see, for example, Haieck et al. (1992), Babu et al. (1992), Martin et al. (1992), and Maune et al. 1992)]. There are fewer reports concerning the +Y liganding position. In troponin C (TnC), the +Y residue is aspartate in sites I and II, but asparagine in sites III and IV. A mutant TnC with asparagine also in loop II has recently been characterized in terms of the stoichiometry of calcium binding (Babu et al., 1992), but unfortunately the effect on calcium affinity was not measured. Studies of synthetic 13-residue peptide analogues of TnC site III indicate that the substitution of Asn by Asp can lead to a slight reduction in the affinity for lanthanum, which was used as a calcium substitution probe (Marsden et al., 1988).

The present investigation concerns the aspartate residue that provides one carboxylate oxygen ligand in position +Y of calmodulin. The role of the second carboxylate oxygen, which is not coordinating to calcium, is investigated by substituting asparagine for the +Y aspartate. One or both of site II in the N-terminal domain and site III in the C-terminal domain have been mutated. Measurements of macroscopic calcium-binding constants for both the intact calmodulins and their tryptic fragments have facilitated a detailed evaluation of the opposite effects of these substitutions on both Ca^{2+} affinity and cooperativity.

MATERIALS AND METHODS

Expression of Calmodulin in Escherichia coli from a Synthetic Gene. A synthetic gene encoding wild-type bovine calmodulin was constructed from overlapping oligonucleotides, essentially as described for bovine calbindin D_{9K} (Brodin et al., 1986). The amino acid sequence of calmodulin and the designed nucleotide sequence are displayed in Figure 3. To construct calmodulins with amino acid changes in Ca^{2+}-binding sites II and III, the gene segment between the *SalI* and *PstI* restriction enzyme sites was replaced by a polylinker, thus facilitating the reassembly of the segment from a set of oligonucleotides containing the mutant sequence as described for calbindin D_{9K} (Linse et al., 1987).

For each of the mutants D58N and D95N, the aspartate codon, GAT, was changed to an asparagine codon, AAC, and in the double-mutant D58N+D95N both of these changes were made. The nucleotide sequences of the assembled genes were confirmed by dideoxy sequencing. The calmodulin expression vector pRCAM was obtained by cloning the complete gene into the runaway plasmid pRCB1 (Brodin et al., 1989). Cells were grown at 30 °C in LB media to an A_{600} of 0.5 and were then diluted to an A_{600} of 0.05 in 400 mL of the same medium prewarmed to 37 °C. IPTG was added to a final concentration of 0.5 mM. The cells were harvested by centrifugation when growth had stopped at an A_{600} of about 0.8.

Purification of Calmodulin and Tryptic Fragments. The *E. coli* cells were sonicated, followed by purification of the expressed calmodulin by ion exchange chromatography, followed by gel filtration and affinity chromatography essentially as described (Jamieson & Vanaman, 1979). Proteolytic fragmentation of calmodulin with trypsin was performed according to Dabikowski et al. (1977). Digestion of calmodulin results in the fragments TR_1C and TR_2C, which are made up of residues 1–74 and 78–148, respectively. The fragments were separated from non-degraded calmodulin by gel filtration and were further purified using a Phenyl–Sepharose column (Andersson et al., 1983, Vogel et al., 1983).

The purities of intact calmodulins and the tryptic fragments were determined by SDS–polyacrylamide gel electrophoresis, agarose gel electrophoresis, and amino acid composition. ^1H NMR[1] spectroscopy was used to verify that the samples were free from EDTA. The absorbance difference between calcium-free and calcium-saturated quin 2 was utilized to determine the residual calcium concentration in each sample (Linse et al., 1991a).

^1H NMR. One-dimensional ^1H NMR spectra were obtained at 500.13 MHz on a GE-OMEGA 500 spectrometer at room temperature and neutral pH.

Ca^{2+}-Binding Constants. Macroscopic calcium-binding constants were obtained from titrations in the presence of a chromophoric chelator, 5,5′-Br_2BAPTA (Linse et al., 1988, 1991a), at room temperature in 2 mM Tris-HCl buffer at pH 7.5. Three individual titrations were performed on each protein or fragment. The values of the macroscopic binding constants were obtained from least-squares fits directly to the measured data as described (Linse et al., 1991a). The total free energy of binding two (for fragments) or four (for intact proteins) calcium ions was calculated from the macroscopic binding constants as $\Delta G_{tot} = -RT \ln (K_1K_2)$ and $\Delta G_{tot} = -RT \ln (K_1K_2K_3K_4)$, respectively. In the case of the fragments, the

[1] Abbreviations: 5,5′-Br_2BAPTA, 1,2-bis(2-amino-5-bromophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; NMR, nuclear magnetic resonance; quin 2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]-methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline.

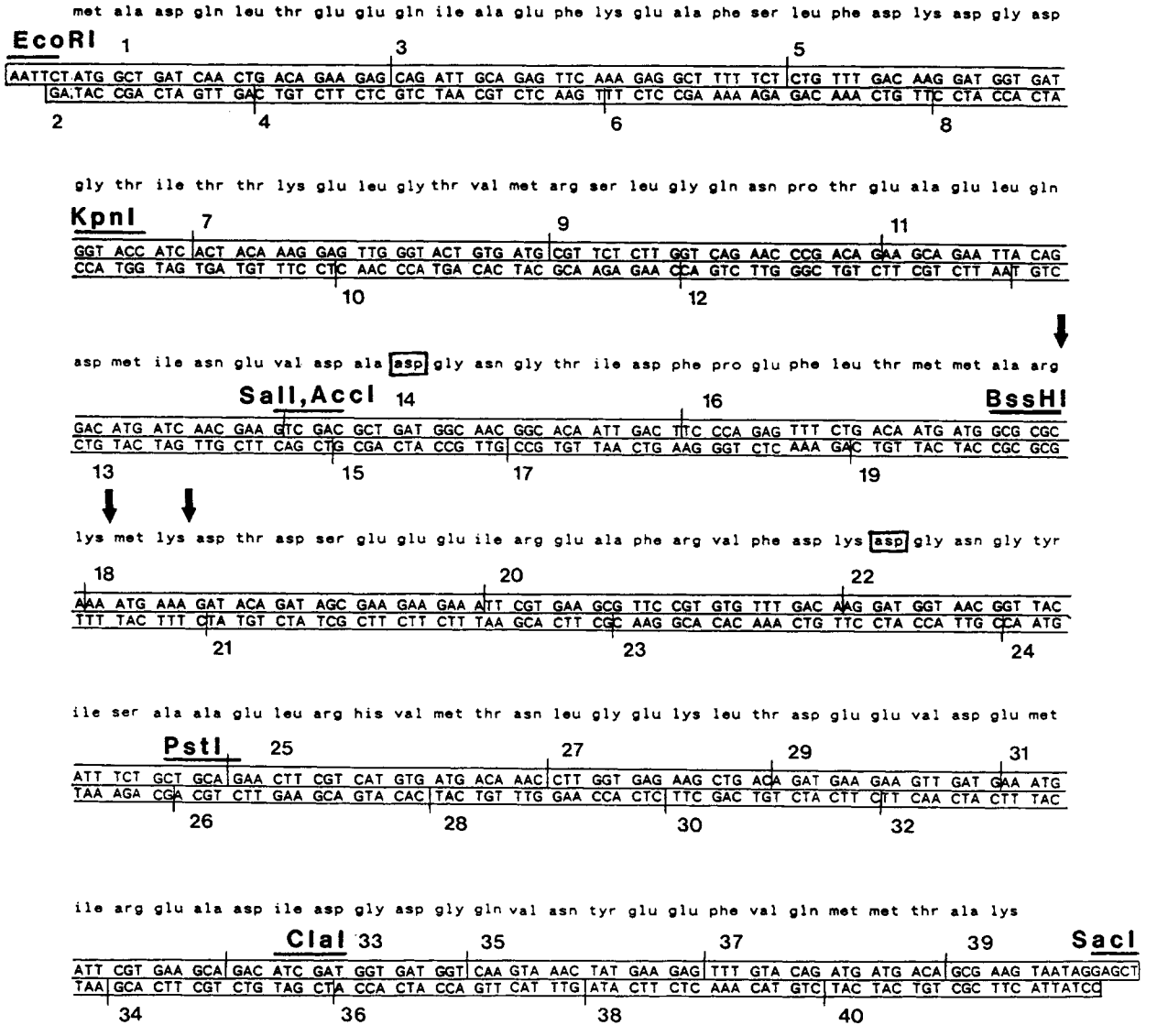

FIGURE 3: Amino acid sequence of wild-type calmodulin and the nucleotide sequence of the synthetic gene. The borders of the overlapping oligonucleotides as well as the restriction enzyme sites used for the construction of the gene are indicated. Aspartates 58 and 95 are boxed in the amino acid sequence, and the arrows indicate the trypsin cleavage sites.

cooperativity of calcium binding can be characterized by $\Delta\Delta G$, the free energy of interaction between the sites, defined as

$$\Delta\Delta G = -RT \ln (K_{II,I}/K_{II}) = -RT \ln (K_{I,II}/K_I) \quad (1)$$

In the above equation, K_I and K_{II} are the site binding constants of sites I and II, respectively, when the other site is empty, and $K_{I,II}$ and $K_{II,I}$ are the corresponding site-binding constants when calcium is already bound to the other site. The present titration technique cannot yield site binding constants. However, $-\Delta\Delta G$ can be rewritten as

$$-\Delta\Delta G = RT \ln (4K_2/K_1) + RT \ln ((\eta + 1)^2/4\eta) \quad (2)$$

where η is the ratio of the affinities of the two sites ($\eta = K_{II}/K_I$), and the factor of 4 is a simple statistical factor that derives from the definition of macroscopic binding constants. As the latter term has a minimum of zero for $\eta = 1$, the macroscopic binding constants can be used to calculate a lower limit to the cooperativity as

$$-\Delta\Delta G_{\eta=1} = RT \ln (4K_2/K_1) \quad (3)$$

It follows directly from eqs 2 and 3 that

$$-\Delta\Delta G_{\eta=1} = -\Delta\Delta G - RT \ln ((\eta + 1)^2/4\eta) \quad (4)$$

The average values, $\bar{x}$, and standard deviations, S, of each parameter ($\lg K_1$, $\lg K_2$, ΔG_{tot}, and $-\Delta\Delta G_{\eta=1}$) were based on three individual titrations on each mutant or wild-type fragment ($\lg K$ stands for $^{10}\log K$). For each parameter (for a given fragment), they were calculated as

$$\bar{x} = \sum_{i=1}^{3}(x_i/\alpha_i^2)/\sum_{i=1}^{3}(1/\alpha_i^2)$$

$$S = [\sum_{i=1}^{3}((x_i - \bar{x})^2/\alpha_i^2)/2\sum_{i=1}^{3}(1/\alpha_i^2)]^{1/2}$$

The x_i's are the parameter values ($\lg K_1$, $\lg K_2$, ΔG_{tot}, or $-\Delta\Delta G_{\eta=1}$) obtained in the individual titrations. The weight of each individual value, $1/\alpha_i^2$, was set so that the range $x_i \pm \alpha_i$ comprises the values of the particular parameter that give twice the sum of the squares of residuals (χ^2) of the optimal fit (when all other parameters were allowed to adjust their values to find the lowest possible χ^2 for each value of x_i).

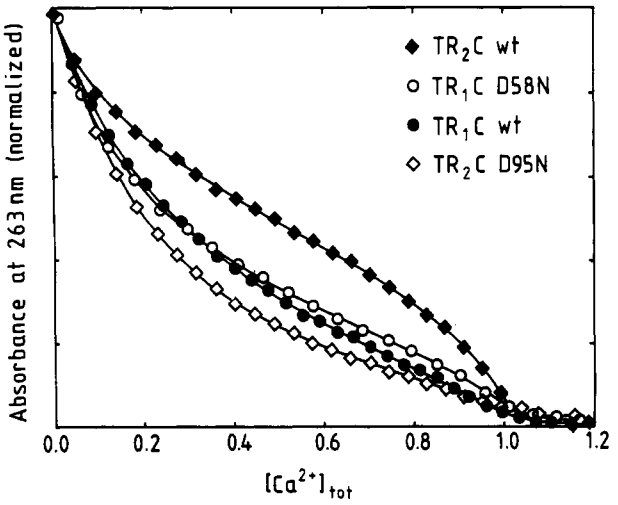

FIGURE 4: Absorbance at 263 nm vs total calcium concentration ($[Ca^{2+}]_{tot}$) from titrations in the presence of 5,5′-Br_2BAPTA for (●) TR_1C wt, (○) TR_1C D58N, (◆) TR_2C wt, and (◇) TR_2C D95N. The optimal curves obtained by least-squares fitting to the data points are shown. Absorbances and calcium concentrations have been normalized in this figure to facilitate comparisons. $[Ca^{2+}]_{tot}$ is normalized so that 1.0 corresponds to the chelator concentration plus 2 times the fragment concentration.

For example, when evaluating the weights for ΔG_{tot}, least-squares fits were performed with different fixed values of the product K_1K_2, and all other parameters, including the individual values of K_1 and K_2, were adjustable. Similarly, for $-\Delta\Delta G_{\eta=1}$ the ratio K_2/K_1 was fixed, while all other parameters, including the individual values of K_1 and K_2, were adjustable.

The averages and standard deviations of ΔG_{tot} for the intact calmodulins were calculated from three titrations exactly as for the fragments. For each individual titration, the α_i values were evaluated from least-squares fits performed for different fixed values of the product $K_1K_2K_3K_4$. All other parameters were adjustable, including the individual values of K_1, K_2, K_3, and K_4.

Confidence limits (95%) can be obtained as $4.3S$.

RESULTS

Purification of Calmodulin and Tryptic Fragments. Expression of wild-type and mutant calmodulins from synthetic genes resulted in a yield of 40 mg of purified protein per liter of *E. coli* culture. The purified calmodulin with the wild-type sequence was indistinguishable from bovine calmodulin when compared in an ATPase activation assay performed as described by Niggli et al. (1979, 1981). The one-dimensional ^1H NMR spectra of these calmodulins were also indistinguishable (data not shown). Proteolytic fragmentation of 100 mg of calmodulin yielded 25 mg of the TR_1C fragment and 28 mg of the TR_2C fragment.

Calcium Binding to Tryptic Fragments. A detailed analysis of the macroscopic calcium binding constants, K_1 and K_2, of the wild-type (wt) and mutant tryptic fragments was made. In free energy terms, the Ca^{2+}-binding process can be described by the total free energy change on binding of two Ca^{2+} ions, $\Delta G_{tot} = -RT \ln (K_1K_2)$, and a lower limit of the free energy of interaction between the two sites, $-\Delta\Delta G_{\eta=1} = RT \ln (4K_2/K_1)$. Figure 4 shows one Ca^{2+} titration in the presence of 5,5′-Br_2BAPTA for each of TR_1C wt, TR_1C D58N, TR_2C wt, and TR_2C D95N. The substitution of asparagine for the aspartate in position +Y in site II (TR_1C D58N) causes a slight change of the titration curve, whereas the corresponding mutation in site III (TR_2C D95N) results in a remarkable difference relative to the wild type. The average values of ΔG_{tot} and $-\Delta\Delta G_{\eta=1}$ for each fragment and the standard deviations in these averages are listed in Table I. Evidently the D58N mutation causes a small but significant increase in $-\Delta G_{tot}$ and a large increase in $-\Delta\Delta G_{\eta=1}$ for TR_1C. In contrast, the D95N mutation leads to a drastic reduction of $-\Delta G_{tot}$ and also lowers $-\Delta\Delta G_{\eta=1}$ for TR_2C.

Table I: Macroscopic Binding Constants of TR_1C and TR_2C, the Free Energy of Binding Two Ca^{2+} ions (ΔG_{tot}), and a Lower Limit to the Free Energy of Site–Site Interaction ($-\Delta\Delta G_{\eta=1}$)[a]

fragment	$\lg K_1$	$\lg K_2$	ΔG_{tot}[b] (kJ mol^{-1})	$-\Delta\Delta G_{\eta=1}$[c] (kJ mol^{-1})
TR_1C wt	6.50 ± 0.04	6.60 ± 0.04	−74.7 ± 0.1	3.9 ± 0.3
TR_1C D58N	6.19 ± 0.05	7.06 ± 0.03	−75.6 ± 0.1	8.4 ± 0.4
TR_2C wt	6.45 ± 0.05	7.45 ± 0.05	−79.3 ± 0.3	9.3 ● 0.7
TR_2C D95N	6.15 ± 0.02	6.56 ± 0.03	−72.5 ± 0.3	5.7 ± 0.1

[a] The standard deviation in each parameter is given as $\pm S$. [b] $\Delta G_{tot} = -RT \ln (K_1K_2)$. [c] $-\Delta\Delta G_{\eta=1} = RT \ln (4K_2/K_1)$.

The two macroscopic binding constants for each fragment and their standard deviations are included in Table I. The data show that the K_1 values are similar for the N- and C-terminal halves, and also that the K_1 values of the halves are similarly affected by the mutations. In contrast, the mutations of sites II and III have opposite effects on the second macroscopic binding constant of the N- and C-terminal halves, respectively.

The results we present here for the tryptic fragment TR_1C wt of recombinant calmodulin are indistinguishable from those previously published for TR_1C from bovine calmodulin (Linse et al., 1991a). For TR_2C, on the other hand, the recombinant wild-type variant appears to bind calcium with a slightly lower affinity than that of the bovine source ($\Delta G_{tot} = -79.3 \pm 0.3$ and -83.4 ± 0.2 kJ mol^{-1}, respectively). The only difference between the two is that the lysine residue in position 115 is trimethylated in bovine TR_2C. The total charge of the recombinant TR_2C is thus one unit less negative.

Calcium Binding to Intact Calmodulins. The calcium-binding processes for the intact wild-type and mutant calmodulins were assessed with the same technique as used for the fragments. However, the data analysis is more complicated due to the presence of four sites in each molecule. The situation in the wild type is such that the average calcium affinity for the C-terminal sites is roughly 6-fold higher than that for the N-terminal sites. This means that the titration curve for the wild type shows two consecutive processes, each of which corresponds to the binding of two calcium ions with positive cooperativity (Linse et al., 1991a). However, for the mutant forms of intact calmodulin, the difference in calcium affinity between the two globular domains is lower than in the wild type. This is especially the case for D95N and the double-mutant D58N+D95N. This means that the two calcium binding events will occur simultaneously. The accuracy of each of the four macroscopic binding constants will therefore be rather low, although the product of all four constants (or $\Delta G_{tot} = -RT \ln (K_1K_2K_3K_4)$) is determined with good precision. The titration data for the intact calmodulins are thus only evaluated in terms of ΔG_{tot}. The average values and standard deviations of ΔG_{tot} (based on three titrations) are summarized in Table II, along with the sum of the values for the corresponding fragments. The data show that the free energy of binding four calcium ions to each intact calmodulin can be very closely reproduced by summing the free energies of binding two calcium ions to the two fragments that constitute its globular domains. Thus, in all four of the intact calmodulins

Table II: Free Energy of Binding Four Ca^{2+} Ions (ΔG_{tot}) to Wild-Type and Mutant Calmodulins and the Sum of the ΔG_{tot} Values for the Tryptic Fragments[a]

calmodulin	ΔG_{tot}[b] (kJ mol^{-1})	$\Sigma(TR_1C + TR_2C)\Delta G_{tot}$ (kJ mol^{-1})
wt	−154.6 ± 0.3	−154.0 ± 0.4
D58N	−154.1 ± 0.3	−154.9 ± 0.4
D95N	−149.0 ± 0.6	−147.2 ± 0.4
D58N + D95N	−148.7 ± 0.2	−148.1 ± 0.4

[a] The standard deviation in each parameter is given as ±*S*. [b] $\Delta G_{tot} = -RT \ln (K_1K_2K_3K_4)$.

studied here, the two globular domains appear to bind calcium independently of each other, as previously concluded for bovine calmodulin (Linse et al., 1991a).

DISCUSSION

The presence of four Ca^{2+}-binding sites in calmodulin precludes detailed analysis of the effects of site-specific mutations on the calcium-binding properties. Combined studies of intact calmodulins and isolated globular domains that contain the same mutations are a fruitful way around this problem. The present results show that mutations with moderate effects on calcium binding can be analyzed with high precision in measurements on the tryptic fragments, whereas such effects are more difficult to quantify when working with whole calmodulins.

A general conclusion based on a wealth of experimental studies on calcium-binding proteins is that there is no simple relation between amino acid sequence and Ca^{2+} affinity (McPhalen et al., 1991). It is nevertheless striking that identical mutations in the structurally very related loops II and III of calmodulin (Figure 2), Asp to Asn in the +Y ligand position, can have opposite effects on the calcium binding properties. The D58N mutation leads to a minor increase in affinity for the two sites in the N-terminal domain and a major increase in cooperativity. The D95N mutation, on the other hand, is found to drastically reduce the Ca^{2+} affinity for the sites in the C-terminal domain and also appears to lower the cooperativity between these two sites. Observed effects on $-\Delta\Delta G_{\eta=1}$ can, in principle, be caused by changes in either the cooperativity, the $-\Delta\Delta G$, or the relative affinities of the sites (cf. eq 4 in Materials and Methods). ^1H NMR data for Ca^{2+} titrations of tryptic fragments from bovine calmodulin have indicated that in each fragment the two sites have similar affinities (Thulin et al., 1984). The large increase in $-\Delta\Delta G_{\eta=1}$ observed for TR_1C D58N must therefore reflect a large increase in $-\Delta\Delta G$, since changes in η from near unity would instead lower $-\Delta\Delta G_{\eta=1}$. A reduction in $-\Delta\Delta G_{\eta=1}$ of the same magnitude observed for TR_2C D95N could reflect a decrease in $-\Delta\Delta G$, but could also arise from a 10-fold increase in η. It is presently not possible to predict, on electrostatic grounds, how the calcium affinity for a given site would change in response to altered charge on a calcium ligand in the same site. It is therefore not possible to speculate on how the observed reduction in K_1 ($=K_I + K_{II}$) is distributed between separate sites. Nevertheless, removal of the charge on the +Y ligand in loop III clearly does not increase the cooperativity, as it does in loop II.

Only one of the carboxylate oxygens of the +Y ligand is considered to directly coordinate the calcium ion. If the only role of the other carboxylate oxygen was purely electrostatic attraction of the calcium ion, then a similar reduction in calcium affinity would be expected in all loops upon substitution of Asn for Asp in position +Y. The different effects observed for the mutations in sites II and III, respectively, thus imply that the involvement of the +Y ligand in interactions other than direct calcium attraction and calcium coordination is of considerable importance for the calcium-binding process. The results also imply that these other interactions differ between the sites. In this context, it is interesting to consider the values of each macroscopic binding constant and how these are affected by the mutations. The fact that the Asp to Asn mutation leads to a very similar decrease in K_1 in both domains indicates that the negative charge of the +Y Asp is important for attracting the first calcium ion. The large differences between the effects on calcium binding caused by the D58N and D95N mutations lie entirely in their opposite effects on K_2. This suggests that the involvement of the non-Ca^{2+}-coordinating oxygen in other types of interactions, such as the hydrogen bond network, is primarily important for mediating the cooperativity.

The large increase observed in the cooperativity between the two sites in the N-terminal domain of calmodulin for the loop II mutation (D58N) shows that it is definitely possible to create cooperativity by neutralizing a charged side-chain ligand in one of the sites. Studies of fragments of bovine calmodulin show that the cooperativity between the sites in TR_1C is also substantially increased when the ionic strength is raised (from $-\Delta\Delta G_{\eta=1} = 3.3 \pm 0.5$ at low ionic strength to ≥10 at 0.15 M KCl; Linse et al., 1991a). Mutational elimination of the charge and an increase in the ionic strength of the surrounding medium are thus both effective in increasing the cooperativity in this domain.

The negative charge on the +Y ligand in loop II appears to counteract cooperativity. It is tempting to speculate whether the role of the +Y side chain in cooperativity is related to the nature of the side chain in position 9 (−X). Both the +Y and -X side chains are hydrogen bonded to the same water molecule (the calcium ligand in position −X; cf. Figure 2). In site II, both of these side chains are negatively charged and part of their mutual interaction is repulsive, which might explain why a neutralization of the +Y side chain favors cooperativity. In site III, on the other hand, there is a serine in position −X, which might explain why high cooperativity can be achieved with aspartate in position +Y of this loop.

The results show that the charge of a ligand residue plays an important role in the cooperativity of calcium binding to calmodulin. We have previously shown that reduction of the cooperativity in calbindin D_{9K} can be achieved by mutations which neutralize negatively charged residues that are not calcium ligands (Linse et al., 1991b). Thus, it may be suggested that the specific distribution of charged and polar residues in and around the calcium sites in an EF-hand pair is a basic requirement for positive cooperativity of calcium binding.

The present work illustrates that characterization of site-specific mutants with altered cooperativity is one fruitful way of finding factors that contribute to the positive cooperativity of calcium binding in EF-hand pair domains. Another approach to the molecular mechanisms of cooperativity comprises extensive multinuclear NMR studies of calbindin D_{9K} in three different forms: with no metal ions bound, with two calcium ions bound, and with a cadmium ion in only one of the sites (Akke et al., 1991; Skelton et al., 1992). On the basis of these studies, it has been proposed that change in dynamics on ligand binding is one of the factors that contributes to the cooperativity (Akke et al., 1991).

In summary, we have shown that the same mutation involving a calcium ligand in an EF-hand can have very

different effects in separate sites both on calcium affinity and cooperativity. This suggests that involvement of a liganding side chain in interactions other than direct calcium coordination is of considerable importance for the calcium-binding process, particularly for its cooperativity.

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