Trifluoperazine Binding to Mutant Calmodulins†

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ABSTRACT: Trifluoperazine (TFP) binding by 14 calmodulins, including 12 produced by site-directed mutagenesis, was determined. While vertebrate calmodulin binds 4.2 ± 0.2 equiv of TFP, *Escherichia coli* expressed but unmutated calmodulins bind about 5.0 ± 0.5 equiv of TFP. The cause for this difference is not known. The *E. coli* expressed proteins consist of two different series expressed from different calmodulin genes, CaMI and SYNCAM. The wild-type genes code for proteins that differ by nine conservative amino acid substitutions. Both these calmodulins bind 5 equiv of TFP with similar affinities, thus none of these conservative substitutions has any additional effect on TFP binding. Some altered calmodulins (deletion of EE^{83-84} or $SEEE^{81-84}$, changing $DEE^{118-120} \rightarrow KKK$, $M^{124} \rightarrow I$, $E^{120} \rightarrow K$, or $E^{82} \rightarrow K$) have no appreciable effect on TFP binding. Other mutations affect either the binding of one TFP (deletion of E^{84}) or about two TFP (changing $E^{84} \rightarrow K$, $EEE^{82-84} \rightarrow KKK$, $E^{67} \rightarrow A$, $DEQ^{6-8} \rightarrow KKK$, or $E^{11} \rightarrow K$). The mutations that affect TFP binding are localized to three regions of calmodulin: The amino-terminal α-helix, the central helix between the two globular ends of calmodulin, and a calcium-binding site in the second calcium-binding domain. The results are consistent with each of these regions either directly participating in drug binding or involved structurally in maintaining or inducing the correct conformation for TFP binding in the amino-terminal half of calmodulin.

Calmodulin binds Ca^{2+} and in its calcium form activates numerous cellular enzymes and structural proteins (Cheung, 1980; Manalan & Klee, 1984; Van Eldik & Watterson, 1985). Also in the presence of Ca^{2+}, calmodulin binds trifluoperazine and other phenothiazine drugs and this binding antagonizes enzyme activation (Weiss & Levin, 1978).

Calmodulin's primary (Watterson et al., 1980) and tertiary structure (Babu et al., 1985; Kretsinger et al., 1986) has been reported. Calmodulin is composed of four domains that are homologous to one another and each domain has a single calcium binding site. The first domain is most similar to the third and the second is most similar to the fourth. This internal homology probably arose by at least two sequential duplications of a gene originally coding for a single calcium-binding domain protein (Watterson et al., 1980). In the crystal structure (Babu, et al., 1985; Kretsinger et al., 1986), calmodulin is shaped roughly like an asymetric dumbbell with two calcium-binding sites in each globular lobe of the molecule and a central α-helix separating the two globular lobes. The globular ends each posses a hydrophobic core, which is accessible to solvent along the surface facing the long helix (Kretsinger et al., 1986). It has been proposed that it is this hydrophobic region at each end of the molecule that interacts with both enzymes and antagonists such as TFP[1] (Kretsinger et al., 1986; LaPorte et al., 1980; Tanaka & Hidaka, 1980; Faust et al., 1987; Massom et al. 1990).

At near physiological ionic strengths and pH, vertebrate calmodulin binds 4–7 equiv of trifluoperazine with micromolar affinity (Massom et al., 1990; Jackson & Puett, 1986). Under similar conditions, 5–6 equiv of chlorpromazine are bound with somewhat lower affinity (Marshak et al., 1985). The location of these drug-binding sites has been investigated by affinity labeling with reactive phenothiazine analogues (Faust et al., 1987; Newton & Klee, 1984; Lukas et al., 1985; Jackson & Puett, 1984), by differential trace acetylation (Giedroc et al., 1985), ^1H NMR (Klevit et al., 1981; Krebs & Carafoli, 1982; Dalgarno et al., 1984), and ESR (Jackson & Puett, 1984). K^{21}, K^{75}, and K^{148} are all implicated as being near one or more of the TFP-binding sites. Studies from several laboratories have demonstrated that there are drug-binding sites in each half of calmodulin (Faust et al., 1987; Lukas et al., 1985; Jackson & Puett, 1984; Giedroc et al., 1985; Newton et al., 1984). These studies have led to various models for the locations of the TFP-binding sites (Faust et al., 1987; Massom et al., 1990; Lukas et al., 1985; Jackson & Puett, 1984; Giedroc et al., 1985; Klevit et al., 1981; Krebs & Carafoli, 1982; Dalgarno et al., 1984). Recently, high-pressure liquid chromatography binding assays have been developed for the study of phenothiazine binding to microgram quantities of calmodulin (Massom et al., 1990; Marshak et al., 1985). These assays are well suited to the study of drug binding to the various calmodulins now being generated by site-directed mutagenesis. Such studies should provide further details about the location of calmodulin's drug-binding sites. Here we present a screen of 14 altered calmodulins.

Methods

Trifluoperazine binding was assayed by using the automated HPLC method previously described (Massom et al., 1990). The calmodulin samples were diluted to 30 μM in buffer A (50 mM MES, 150 mM KCl, 1.0 mM $CaCl_2$, pH 6.5) and 10 μL was injected for each binding determination. The

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[1] Abbreviations: TFP, trifluoperazine dihydrochloride; CaM, calmodulin; HPLC, high-pressure liquid chromatography.

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Table I[a]

name	region mutated	change	TFP binding n_{app}	TFP binding K_{app} (μM)
CaM	none	none	4.2 ± 0.2	6 ± 1
CaMI	none	none	5.0 ± 0.2	23 ± 5
CaMI-1	E^{84}	delete	3.7 ± 0.2	12 ± 6
CaMI-2	EE^{83-84}	delete	5.4 ± 0.8	9 ± 1
CaMI-3	$SEEE^{81-84}$	delete	4.4 ± 1.1	9 ± 9
SYNCAM-1	none	none	5.0 ± 0.5	6 ± 2
SYNCAM-8	EEE^{82-84}	KKK	2.7 ± 0.2	4 ± 1
SYNCAM-12	$DEE^{118-120}$ M^{124}	KKK I	4.6 ± 0.4	3 ± 0.5
SYNCAM-13	E^{67}	A	2.3 ± 0.0	5 ± 0.1
SYNCAM-18	EEE^{82-84}, $DEE^{118-120}$,M^{124}	KKK KKK, I	3.0 ± 0.2	10 ± 0.2
SYNCAM-24	DEQ^{6-8}	KKK	1.9 ± 0.4	9 ± 7
SYNCAM-26	E^{11}	K	2.5 ± 0.2	7 ± 1
SYNCAM-28	E^{84}	K	3.0 ± 1.3	8 ± 3
SYNCAM-29	E^{120}	K	5.2 ± 0.3	12 ± 0
SYNCAM-39	E^{82}	K	4.9 ± 0.8	8 ± 6

[a] K_{app} and n_{app} were derived from Scatchard plots of each data set. The values given are the mean ± the standard deviation obtained by averaging the values obtained from the Scatchard plot for two or more complete data sets.

HPLC method used gives data on the binding of TFP at eight different concentrations; all injections were made in duplicate. The areas of the peaks obtained in the absence of trifluoperazine were subtracted from the other areas.

Protein concentration was determined by amino acid analysis with β-Ala as an internal standard as previously described (Jarret et al., 1986).

Calmodulins. Porcine brain calmodulin was purified by using melittin–silica as previously described (Rhoades et al., 1988). Site-directed mutagenesis was used to generate the various mutant calmodulins. The mutations are described in Table I. Three of these, the deletion mutants CaMI-1, CaMI-2, and CaMI-3 and the unmutated control, CaMI, were generated and purified as described by Persechini et al. (1989). Another nine mutant calmodulins (SYNCAM-8, -12, -13, -18, -24, -26, -28, -29, and -39) and the unmutated control (SYNCAM-1) were generated and purified by procedures similar to those previously described (Craig et al., 1987; Roberts et al., 1985). Mutant calmodulins in this series were previously referred to by using the "VU" prefix (Craig et al., 1987; Roberts et al., 1985) but more recently have been named "SYNCAM" (Weber et al., 1989). All calmodulins were homogeneous as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Results and Discussion

The binding data obtained with the various proteins are shown in Figure 1; Figure 1A summarizes the results obtained with the CaMI proteins, Figure 1B shows those SYNCAM mutants that had no appreciable effect on TFP binding, and those SYNCAM mutants that affected TFP binding are shown in Figure 1C. The identity of the various mutants is shown in Table I along with the apparent number of TFP-binding sites (n_{app}) and apparent affinities (K_{app}) found from the Scatchard plots of the data. Figure 2 gives a pictorial representation of vertebrate calmodulin showing the location of the various alterations in the various proteins.

Limitation of the Approach Use. The smallest data set analyzed for any calmodulin contains two separate binding experiments and includes 32 separate measures of TFP binding at 16 different TFP concentrations. Most calmodulins were studied even more extensively. While this extensive analysis leaves little doubt about the veracity of the results presented,

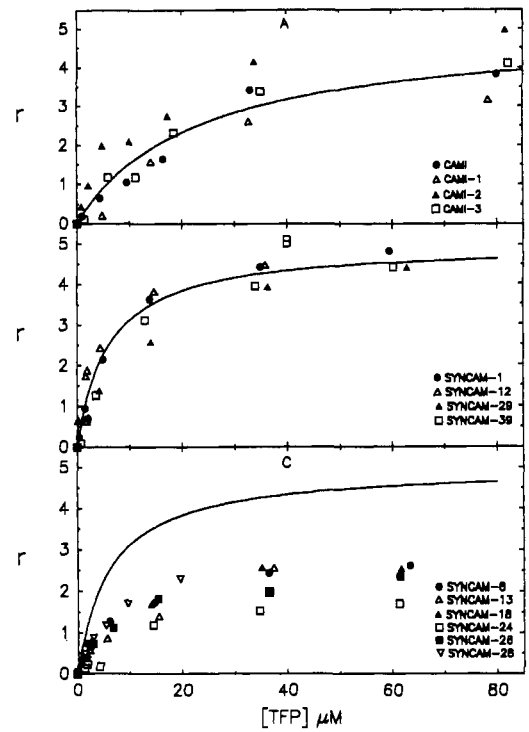

FIGURE 1: TFP-binding data obtained for the calmodulins. "r" is mol of TFP bound/mol of calmodulin. The data shown are the mean for duplicate determinations from a typical data set. (A) The CaMI proteins. The line shown was derived for the Scatchard equation with $n = 5.0$ and $K = 23$ μM, which are the derived values for the CaMI protein. (B) SYNCAM mutants that showed no change in TFP binding. These calmodulin mutants were not different from the control SYNCAM-1. The line shown is from the Scatchard equation for $n = 5$ and $K = 6$ μM, which are the derived values for the SYNCAM-1 control. (C) SYNCAM mutants whose TFP binding was affected. The line shown is the same as in (B), i.e., the equation for the SYNCAM-1 control.

there are some limitations. The highest TFP concentration used in these experiments was 150 μM and the data were analyzed by using the Scatchard equation, which was previously shown to adequately describe the binding of TFP to calmodulin (Massom et al., 1990). However, a result of this analysis is that any mutation that lowers the TFP-binding affinity of one or more sites by more than 1 order of magnitude would be reflected in a reduction in the number of apparent binding sites. Thus, the K_{app} and n_{app} reported are only apparent values; they should not be interpreted as strict thermodynamic values. This treatment is adequate for the purpose of discovering which mutations affect TFP binding.

Since a given alteration in calmodulin may either act locally or have far-ranging effects on structure, the results obtained are interpreted cautiously.

Comparison of Wild-Type Calmodulins. Vertebrate calmodulin consistently shows the presence of 4.2 ± 0.2 TFP binding sites, in agreement with our previous report (Massom et al., 1990), but the control proteins SYNCAM-1 and CaMI consistently showed five apparent binding sites (Table I). This was confirmed by interspersing determinations on the vertebrate protein as a control during the studies of the *Escherichia coli* expressed proteins and using amino acid analyses to confirm that the protein concentrations were accurate. All of the TFP binding was Ca^{2+}-dependent, including the extra binding of the *E. coli* expressed proteins. For example, at 120 μM TFP less than 7% of the Ca^{2+}-dependent TFP binding of CaMI was observed when EGTA replaced Ca^{2+}. The *E. coli* expressed controls (SYNCAM-1 and CaMI) share three differences from the vertebrate calmodulin: (1) both have a

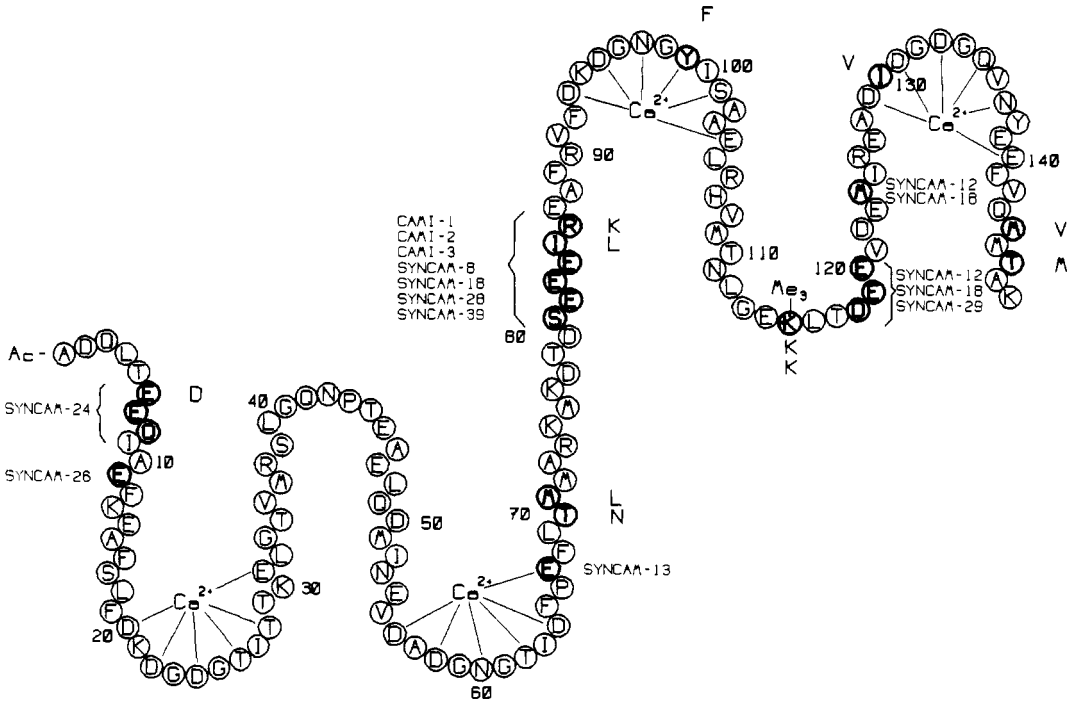

FIGURE 2: Positions of the alterations in calmodulin. The sequence of vertebrate calmodulin is shown with the calcium-binding sites (Watterson et al., 1980). On the basis of the crystal structure (Babu et al., 1985; Kretsinger et al., 1986) those regions that are α-helical are shown by staggering the symbols. Those residues that differ in one or more protein are shaded. From the amino terminus, the positions of the mutations are shown to the left. To the right, residues that are different between vertebrate calmodulin and CaMI are shown closest to the chain and those different in SYNCAM-1 are more distant.

nonacetylated amino terminus, (2) both contain lysine rather than trimethyllysine at position 115, and (3) both were isolated by using low-pressure chromatography on phenyl-Sepharose, while the vertebrate calmodulin was isolated by using HPLC on melittin–silica. Vertebrate calmodulin isolated by a different procedure (Faust et al., 1987) utilizing TAPP-Sepharose gave virtually identical results with those reported for the melittin–silica-purified vertebrate calmodulin (data not shown). Furthermore, when chicken calmodulin and SYNCAM-1 purified by the same phenyl-Sepharose protocol were compared, the proteins gave n_{app} of 3.8 ± 0.3 and 5.2 ± 0.5, respectively. (K_{app} was 7.0 and 7.6 μM, respectively). These observations suggest that the increased drug-binding stoichiometry does not arise from the way the calmodulins were isolated. Of the two posttranslational differences, only the methylation state of K^{115} has been reported to markedly affect any activity of calmodulin, i.e., the activation of NAD kinase (Roberts et al., 1985). However, as shown below, modifications in the amino-terminal region of calmodulin can have dramatic effects on TFP binding. Thus, posttranslational differences may account for the enhanced TFP binding by the *E. coli* expressed proteins.

SYNCAM-1 versus CaMI. The two genes used to code for these calmodulins give slightly different proteins. The sequence of CaMI is identical with that of vertebrate calmodulin with the exception of the posttranslational modification differences already noted above. However, SYNCAM-1 is a highly engineered gene coding for a sequence that has nine additional differences from vertebrate calmodulin. These are shown in Figure 2. Since both proteins bind the same number of TFPs (Table I), none of these differences affect the number of sites. All of the differences in SYNCAM-1 also occur in various nonvertebrate calmodulins and thus are phylogenetically conservative replacements. The apparent affinity of SYNCAM-1 for TFP is indistinguishable from that of vertebrate calmodulin while CaMI has about 4-fold lower affinity; the reason for this lower affinity is unclear.

Central Helix Mutants. The CaMI mutants all involve deletions in the $SEEE^{81-84}$ region of the central helix of calmodulin and several SYNCAM mutants also involve this region (Table I). The results obtained show the importance of this region, particularly E^{84}. Deletion of E^{84} decreases TFP binding by about 1 equiv (CaMI-1), while changing it to a K decreases the binding by about 2 equiv (SYNCAM-28). Deleting adjacent residues (EE^{83-84}, CaMI-2, or $SEEE^{81-84}$, CaMI-3) apparently restores TFP binding to the level of the control, while changing adjacent residues ($EEE^{82-84} \rightarrow$ KKK, SYNCAM-8) does not significantly differ from changing E^{84} alone (SYNCAM-28); i.e., in either case, about 2 equiv fewer of TFP are bound over the control. The lower TFP binding by SYNCAM-8 is apparently due primarily to the change in E^{84} alone (as in SYNCAM-28) since E^{82} can be changed to K (SYNCAM-39) with no significant effect over the control. This acidic region of sequence may be important to (*1*) the intermolecular interactions between calmodulin and the cationic drug, TFP, or (2) intramolecular interactions within calmodulin neccesary to induce or maintain the conformation that binds TFP. The data presented in Table I suggest that the latter may be more important. Since the binding of two TFP is affected by the $E^{84} \rightarrow$ K mutations (SYNCAM-28), intermolecular interactions would require that two TFP must interact with this residue and yet the protein with deletion of all three Es and an additional S (CaMI-3) binds the same number of TFPs as the control. However, these residues may be conformationally important; one or more of these acidic residues may interact with residues elsewhere in calmodulin and these interactions may be necessary to the correct TFP-binding conformer. E^{84} could be especially important to this interaction and its deletion or alteration prevents correct folding of at least a portion of calmodulin's structure, perhaps localized to one-half of calmodulin. Deleting residues from this helical region moves other acidic residues into the region previously occupied by E^{84} (the adjacent EE^{82-83} or DTD^{78-80}) and allows other interactions to compensate for the removal

of E^{84}. Thus, perturbation of electrostatic interactions may be less in the deletion mutants. The effect of these mutations on enzyme activation has also been measured (Persechini et al., 1989; Craig et al., 1987; Weber et al., 1989) and these results will be discussed further below.

SYNCAM Point and Cluster Mutants Elsewhere in Calmodulin. The SYNCAM mutants not along the central helix of calmodulin provide further insights. Two of these involve the calcium-binding domains of calmodulin. One of these is a consequence of the $Y^{99} \rightarrow F$ change present in all the SYNCAM proteins. This residue occurs at the "-X" coordination position of the third calcium-binding domain at a position where the oxygen coordination is provided by a peptide bond carbonyl or a water molecule and not by the amino acid side chain. Thus, it is perhaps not surprising that a substitution at this position would have no effect on calcium binding by calmodulin. We show here that $Y \rightarrow F^{99}$ has no effect on the apparent number of TFP-binding sites (compare SYNCAM-1 and CaMI, Table I). A $Y \rightarrow F$ change also occurs at the homologous position in troponin C and in the plant calmodulins (Roberts et al., 1985; Collins et al, 1973). Previously, we have shown that troponin C has the same number of TFP-binding sites as does vertebrate calmodulin (Massom et al., 1990), and this supports the observation here that this change does not alter TFP binding.

The other change at a Ca^{2+}-binding site is in SYNCAM-13 ($E^{67} \rightarrow$ Ala at the "-Z" ligand in the second calcium-binding domain). Here the effect was more dramatic with SYNCAM-13, showing less than half the TFP binding of the SYNCAM-1 control (Table I). This coordination position for the Ca^{2+} ion requires one of the oxygens in the side chain of E^{67} and the change to Ala would remove these oxygens and should reduce calcium binding. In a preliminary report (Haiech et al., 1989), this mutation was reported to cause the loss of two calcium binding sites. We previously reported that under the conditions used here, vertebrate calmodulin does not bind TFP in the absence of Ca^{2+} (Massom et al., 1990). The data (Table I) thus suggest that this mutation, because of its effect on calcium binding, abolished TFP binding in the amino-terminal half of calmodulin.

Two other mutants point out the importance of the region from residues 6 to 11 at the amino-terminal end of calmodulin to TFP binding. The mutation of $DEQ^{6-8} \rightarrow$ KKK (SYNCAM-24) or the change $E^{11} \rightarrow K$ (SYNCAM-26) abolishes about half of the TFP-binding sites. This region is α-helical (Babu et al., 1985; Kretsinger et al., 1986) and is rather far removed from any region suspected previously of being involved in TFP binding. For example, affinity labeling and differential acetylation experiments have pointed to the regions near K^{21}, K^{75}, and K^{148} as being important to drug and peptide antagonist binding (Faust et al., 1987; Newton & Klee, 1984; Lukas et al., 1985; Jackson & Puett, 1984; Giedroc et al., 1985), but the involvement of the amino-terminal α-helix had previously gone unsuspected.

Finally, several other mutations have no effect on TFP binding. As summarized in Table I, $DEE^{118-120}$ (SYNCAM-12), E^{120} (SYNCAM-29), and E^{82} (SYNCAM-39, also discussed above) can be changed to K with relative impunity (Table I). It is interesting to note that some of these mutations that had no effect have quite dramatic effects when homologous positions in calmodulin are changed. Thus, $DEE^{118-120}$ (domain IV) is homologous to positions EEE^{82-84} (domain III), but while the first may be changed with no apparent effect (SYNCAM-12, Table I), the latter change (SYNCAM-8) has a dramatic effect on TFP binding. Similarly E^{11} (domain I), E^{84} (III), and E^{120} (IV) are all homologous positions, but the changes in the first two have a large effect while changes in the latter has no effect on TFP binding. Thus, whether or not a mutation at a homologous position will effect TFP binding appears to parallel the similarity between the domains. It appears from this limited data that the effect of a mutation in domain I will also be mimicked by a mutation in the more similar domain III but may be quite different when it occurs in the less similar domains II (or IV). This indicates that the dissimilarity between domains I and II (or III and IV) is important to the way in which TFP binds to calmodulin.

The data are also internally consistent. The mutations in SYNCAM-8 and SYNCAM-12 are added together in SYNCAM-18. Only the changes made in SYNCAM-8 had an effect on drug binding and SYNCAM-18 shows the same drug binding as does SYNCAM-8. Thus, as one would expect, adding together one mutation that effects TFP binding and another that does not gives only the affect of the detrimental mutation. The basic agreement between the CaMI and SYNCAM proteins on the importance of the long helix region EEE^{82-84} also demonstrates the consistency of the observations.

An alternation may be at a TFP-binding site and affect binding directly or it may be distant from a TFP-binding site and exert its effect by inducing or hindering a change in conformation. Hence, we have interpreted the data in the more limited sense of defining which regions of calmodulin are important to TFP binding without discussing where the binding sites may be in relation to the mutations studied. The central helix mutants (CaMI-1, -2, and -3 and SYNCAM-8, -18, -28, and -39) point out the possible pitfalls of interpreting the effect of a mutation too narrowly. The effect of mutation in this region can be to have no effect, affect one binding site, or affect two binding sites depending upon whether a deletion or substitution is made and precisely what the substitution is and where it is made.

Regardless of these limitations, the data presented here are valuable to discovering what elements of calmodulin's structure are important to TFP binding. These may be summarized here:

1. The region $SEEE^{81-84}$ is important to about half of the TFP binding observed. The results obtained are most consistent with this region serving some important function in the conformation of calmodulin.

2. Mutations that alter amino acid side chains involved in coordinating calcium (E^{67}, SYNCAM-13) can affect over half of the TFP-binding to calmodulin. Previously, we had shown that at least three Ca^{2+} ions must be bound to calmodulin if TFP is to bind (Massom et al., 1990). One of these Ca^{2+} ions must bind to the calcium-binding site in domain II. Altering this site probably abolishes all TFP binding in the amino-terminal half of calmodulin. Changes at calcium binding sites that do not affect calcium coordination (the $Y^{99} \rightarrow F$ difference between vertebrate calmodulin and CaMI and the SYNCAM series) are without effect.

3. The dissimilarities noted between the homologous domains of calmodulin are important to TFP binding.

4. The amino-terminal α-helix (specifically sequence positions 6–8 and 11) of calmodulin also serves some important role in the ability to bind TFP.

The effect of some of these mutations on calmodulin's ability to activate enzymes has also been measured. Thus, CaMI and the mutants of it activate phosphodiesterase, calcineurin, myosin kinase, and NAD kinase at least as effectively as does brain calmodulin (Persechini et al., 1989). Over this mutant series, less than an order of magnitude difference was found

in the concentration required to half-maximally activate any of the enzymes; thus, enzyme binding was relatively unperturbed. These results parallel those found with TFP binding here. The largest change was seen with CaMI-1 and its TFP binding was only diminished by about one-fifth; the other proteins gave results quite similar to those of unmutated control and the apparent affinity for TFP changes only about 2-fold over this series (Table I).

Enzyme activation by the SYNCAM-8 mutant shows much larger differences. While SYNCAM-8 ($EEE^{82-84} \rightarrow KKK$) fully activates phosphodiesterase and type II calmodulin-dependent protein kinase with a similar affinity to that obtained with SYNCAM-1, myosin light chain kinase was only activated to about 30% of maximum by SYNCAM-8 and NAD kinase was not activated at all (Craig et al., 1987; Weber et al., 1989). SYNCAM-28 ($E^{84} \rightarrow K$), which has a similar reduced drug-binding capacity, also exhibits altered activation of myosin light chain kinase (Shoemaker et al., 1990). To some extent these differences observed with myosin light chain kinase and NAD kinase parallel the differences found here in SYNCAM-8's TFP binding. Relative to its unmutated control, our results show that half of the TFP binding was abolished by this mutation. In this case, the effect on TFP binding is reflected in the enzyme activation data. On the other hand, a calmodulin mutant in which $DEE^{118-120}$ are changed to KKK exhibits altered activation of myosin light chain kinase and type II calmodulin-dependent protein kinase (Weber et al., 1989), but SYNCAM-12 ($DEE^{118-120} \rightarrow KKK$ and $M^{124} \rightarrow I$) binds TFP as well as the SYNCAM-1 control (Table I). Thus, considering all the data, there is little correlation of enzyme activation and TFP-binding capacity.

These studies involved all together 22 discreet changes in calmodulin (about 15% of the sequence). Fifteen of these changes (nine of which were differences between the SYNCAM-1 protein and vertebrate calmodulin) had no effect on TFP binding. This indicates that many of the differences that have been noted in calmodulins from different species (many of which are present in SYNCAM-1) have no effect on TFP binding. Seven other sequence positions localized to three regions (the amino-terminal region, the central helix, and the calcium-binding site of domain II) are important to half of the TFP-binding capacity of calmodulin. All of the alterations in the carboxyl-terminal lobe of calmodulin that were a part of this study had no effect on TFP binding and so the regions of structure necessary to the TFP binding that occurs there remain to be elucidated.

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