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BINDING OF TRIFLUOPERAZINE AND FLUORENE-CONTAINING COMPOUNDS TO CALMODULIN AND ADDUCTS

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Abstract—Calmodulin can be specifically acylated with a fluorene-containing hydrophobic spin-labeling reagent at just Lys 75 or at Lys 75 and Lys 148. The binding of trifluoperazine to calmodulin and the two adducts was determined using a Hummel–Dreyer procedure, and binding of the phenothiazine was found to be characterized by apparent positive cooperativity and an apparent limiting stoichiometry of about seven binding sites per protein molecule. Two non-reactive fluorene-containing compounds were synthesized, and both reagents exhibited far less binding to calmodulin than did trifluoperazine. One of these was also assayed for binding to the monolabeled adduct, and this binding was about half that observed with calmodulin and was non-cooperative. Thus, the qualitative and quantitative binding parameters of hydrophobic groups to calmodulin can be quite different.

The Ca^{2+}-binding protein calmodulin appears to be involved in a remarkable number of regulatory intracellular processes [1–4]. The recently described crystallographic structure [5] shows that, like troponin C [6, 7], calmodulin has two globular lobes, each containing two Ca^{2+} binding sites, connected by a relatively long α-helix. In addition to the Ca^{2+}-dependent interaction of calmodulin with a variety of proteins, mainly enzymes [1–4], calmodulin binds other compounds, also in a Ca^{2+}-dependent manner, including phenothiazines [8–10] and peptides [11–17]. These latter ligands are of considerable interest since they inhibit the ability of calmodulin to interact with and stimulate the activities of a variety of enzymes that have been examined. Thus, elucidation of the binding nature of these relatively small compounds may reveal to some extent the calmodulin binding sites for target enzymes.

Earlier, we reported that calmodulin can be modified with high specificity using a hydrophobic labeling reagent [18]. The reagent, N^4-(9′-fluorenylmethyloxycarbonyl)-4-amino-1-oxyl-4-succinimidyloxycarbonyl-2,2,6,6-tetramethylpiperidine (**I**, Fig. 1), labeled Lys 75 (1 : 1 adduct) most readily and then, at a slightly reduced rate, labeled Lys 148 (2 : 1 adduct). The labeling could be inhibited by trifluoperazine (**II**, Fig. 1) and is Ca^{2+}-dependent [18]. Thus, it was of interest to investigate the relatedness of the labeled residues to the sites of interaction of an inhibitory compound such as trifluoperazine. Herein, we report that the formation of covalent calmodulin derivatives reduced the affinity of trifluoperazine to the derivatives and that analogs of the initial labeling reagent (**III** and **IV**, Fig. 1) exhibited much more limited binding to calmodulin and the 1 : 1 adduct.

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MATERIALS AND METHODS

Materials. *N*-Fluorenylmethyloxycarboyl-L-alanine *p*-nitrophenylester was from Chemilog (South Plainfield, NJ), ethanolamine was from Eastman (Rochester, NY), and TLC plates were from EM Reagents (Gibbstown, NJ). Buffers and other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or Mallinckrodt (Paris, KY).

Synthesis of **I** *and preparation of adducts.* **I** was synthesized and purified as described earlier [18]. Calmodulin was purified from porcine testes [18], and adducts were prepared by reacting the protein (*ca.* 29 μM) with **I** (*ca.* 63 μM) in a 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer, pH 7.8, containing 2 mM $CaCl_2$, at ambient temperature. As shown previously [18], a 1 : 1 adduct is mainly characterized by a modification of Lys 75 on calmodulin; this adduct was prepared by limiting the reaction time to 12 min. A 2 : 1 adduct is primarily characterized by modification of Lys 75 and Lys 148 [18]; this calmodulin derivative was enriched by extending the reaction time to 25 min. The addition of 0.25 M Tris and 0.8 M glycine terminated the reaction, and the mixture was dialyzed against 25 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl and 0.5 mM EDTA. The solution was then applied to a DE-52 column, equilibrated in the same buffer, which was then developed first with the buffer containing 0.22 M NaCl and next with a convex ionic strength gradient to 0.27 M NaCl. The two adducts are separated under these conditions with the 1 : 1 adduct eluting first. Purity was established by reverse phrase HPLC using a Synchron RP-P column developed with a linear gradient of 20 mM piperidinium

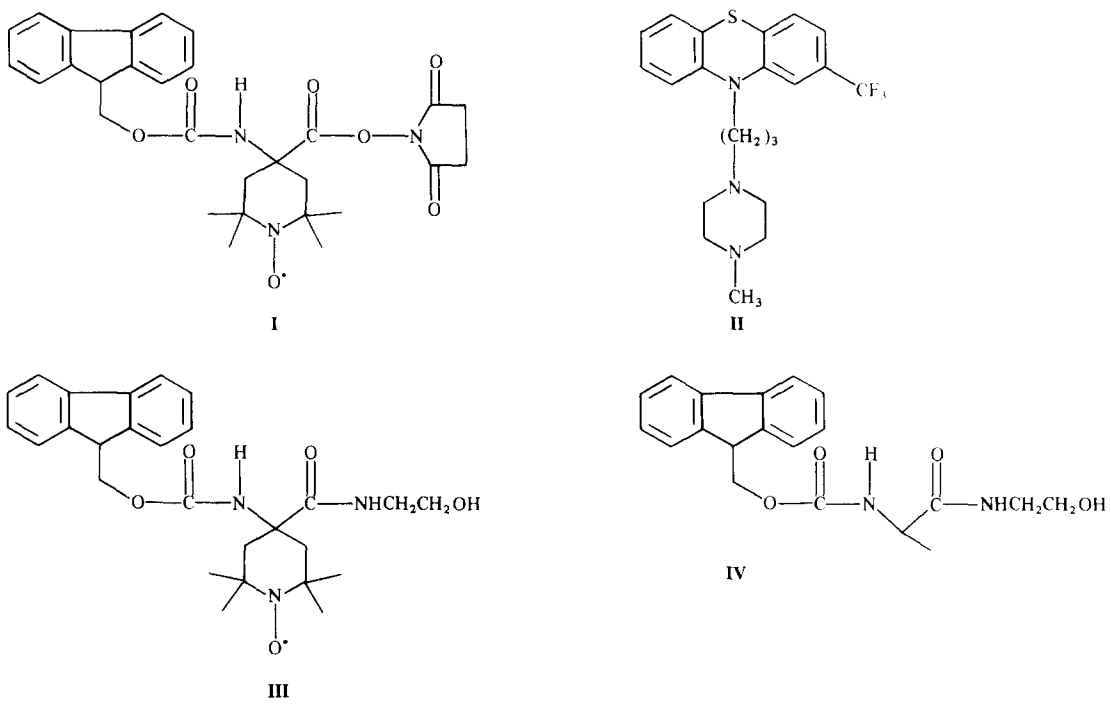

Fig. 1. Structures of compounds used in these studies: (**I**) a spin-labeling reagent, (**II**) trifluoperazine, and (**III** and **IV**) nonreactive analogs of **I**.

acetate, pH 5.6, containing 0.5 mM EDTA and acetonitrile (28 to 40%, then elution at a constant 40%). The purified adducts were dialyzed against water, lyophilized, chromatographed on Sephadex G-50 (50 mM ammonium acetate, pH 6.0), dialyzed against water, and lyophilized.

Synthesis of **III**. To 30 mg (56 μmoles) **I** in 1 ml dichloromethane was added 7 μl ethanolamine (115 μmoles). After 20 min a white precipitate was removed by filtration, and the filtrate was applied to a 2 mm preparative TLC plate and developed using dichloromethane–isopropanol, 93:7. The band with R_f 0.21 was eluted using dichloromethane–isopropanol, 10:3 twice and 1:1 once. The extract was dried by rotary evaporation and crystallized from hot CH_3CN, yielding orange crystals, 16.2 mg (58%): m.p. 155–156°. Calculated for $C_{27}H_{34}N_3O_5$: C 67.5, H 7.1, N 8.7; found: C 67.5, H 7.1, N 8.7.

Synthesis of **IV**. To 112 mg of *N*-fluorenylmethyloxycarbonyl-L-alanine *p*-nitrophenylester stirring in 10 ml dichloromethane was added 30 mg of ethanolamine in 0.5 ml dichloromethane. After 90 min an equal volume of ethanolamine solution was added, and the reaction was continued for another 90 min. During this reaction, the primary TLC spot (10:1 methylene chloride–isopropanol) was converted from R_f 0.95 to R_f 0.21, though some residual starting material remained. Nitrophenol (R_f 0.52) was largely removed by extraction with H_2O. Three recrystallizations from hot ethylacetate effectively removed starting material; yield 75 mg: m.p. 161–162°. Calculated for $C_{20}H_{22}N_2O_4$: C 67.8, H 6.3, N 7.9; found: C 67.7, H 6.6, N 7.4.

Binding studies. The binding experiments were conducted using the method of Hummel and Dreyer [19]. The column, a 4.6 × 250 mm stainless-steel column packed with Bio-Gel P-30, was attached to a Rheodyne injector fitted with a 20 μl sample loop and to a Perkin–Elmer Series 10 pump. The buffer was 25 mM HEPES, pH 7.5, containing 0.2 M NaCl and 1 mM $CaCl_2$; in some experiments 2 mM ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA) was also added. In order to measure the equilibrium binding of trifluoperazine, **III** and **IV** to calmodulin and adducts, the desired concentration of the ligand was added to the equilibrating buffer. The flow rate was 0.1 ml/min, and detection was by u.v. absorption measured at either 255 nm or 305 nm with a Perkin–Elmer LC-75 spectrometer. Integration was done on the ligand trough with a Perkin–Elmer LCI-100 integrator (attached to the detector with analog signal reversed). The response was calibrated by injection of concentrations of ligand which were in excess of the concentration of ligand used to equilibrate the column. Calmodulin or calmodulin adducts were dried *in vacuo* (~30 μg each in multiple 1.5-ml centrifuge tubes), then dissolved several minutes prior to injection in 30 μl of column buffer containing the appropriate ligand concentration, and 20 μl of this solution was injected. The amount of protein injected was determined by amino acid analysis on equivalent aliquots of protein. From the known amount of protein and the integrated area of the trough, it is possible, using the integrator response factor of the fluorene-containing compounds, to determine the moles of bound ligand per mole of

calmodulin or adduct. The area under the peak cannot be used for determination of bound ligand since the protein-associated chromophores of the adducts contribute to the absorbance. Thus, by equilibrating the column at various concentrations of ligand, equilibrium binding isotherms can be constructed. The results are reported as moles ligand bound per mole protein, and this is designated as **r**.

Phosphodiesterase assays. The activity of stimulatable cyclic nucleotide phosphodiesterase was measured using a bovine brain fraction and ^{3}H-labeled cyclic guanosine monophosphate as described earlier [15, 18]. Under the conditions used, the maximal fold-increase in enzymic activity upon the addition of Ca^{2+}-calmodulin was about 4. The results are normalized to 100% for the activity at saturating Ca^{2+} (V_s) and to percent for the basal activity (V_0, i.e. no calmodulin or calmodulin plus EGTA) as follows: $100(V - V_0)/(V_s - V_0)$, where V = measured activity at a given calmodulin or adduct concentration.

RESULTS

Shown in Fig. 2 are typical Hummel–Dreyer gel permeation profiles for injections of a constant amount of calmodulin or calmodulin adducts at a fixed concentration of the equilibrating ligand, trifluoperazine. The peak reflects bound ligand as well as chromophores associated with the calmodulin adducts, and the trough measures the amount of ligand removed from the equilibration buffer by association with the protein. The binding measurement at this fixed concentration of ligand emphasizes the difference in binding characteristics for the two adducts compared to calmodulin: calmodulin bound much more trifluoperazine than the 1:1 adduct, and the 1:1 adduct bound more than the 2:1 adduct.

The binding isotherm (Fig. 3A) also points to differences in trifluoperazine binding characteristics. However, the adduct isotherms demonstrate that covalent labeling with a hydrophobic probe did not eliminate the capacity of calmodulin to bind trifluoperazine, since the adducts bound at least as much phenothiazine as unmodified calmodulin. For calmodulin and for the two adducts, the binding appeared to be cooperative. The Scatchard plots are consistent with apparent positive cooperativity (Fig. 3B), as are the Hill coefficients (2.0, 1.6 and 1.7 for calmodulin (N = 7), the 1:1 adduct (N = 7) and the 2:1 adduct (N = 8), respectively, where N denotes the number of apparent binding sites). The binding midpoints, interpolated from the Hill plots, were 8.0, 11.9 and 15.0 μM for calmodulin, the 1:1 adduct and the 2:1 adduct respectively. The Ca^{2+}-independent (EGTA present) binding of trifluoperazine was greater for the adducts compared to

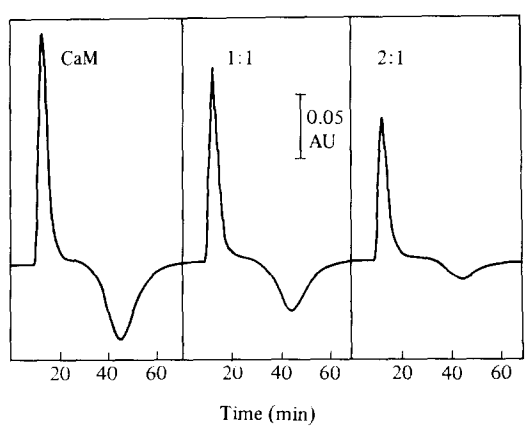

Fig. 2. Hummel–Dreyer gel permeation profiles for calmodulin and the two adducts. The buffer contained 20 μM trifluoperazine, and equivalent amounts (1 nmole) of calmodulin and each adduct were injected. Key: CaM, calmodulin; 1:1, 1:1 adduct of **I** with calmodulin (Lys 75 modified); 2:1, 2:1 adduct of **I** with calmodulin (Lys 75 and Lys 148 modified). The absorbance was measured at 255 nm for all samples.

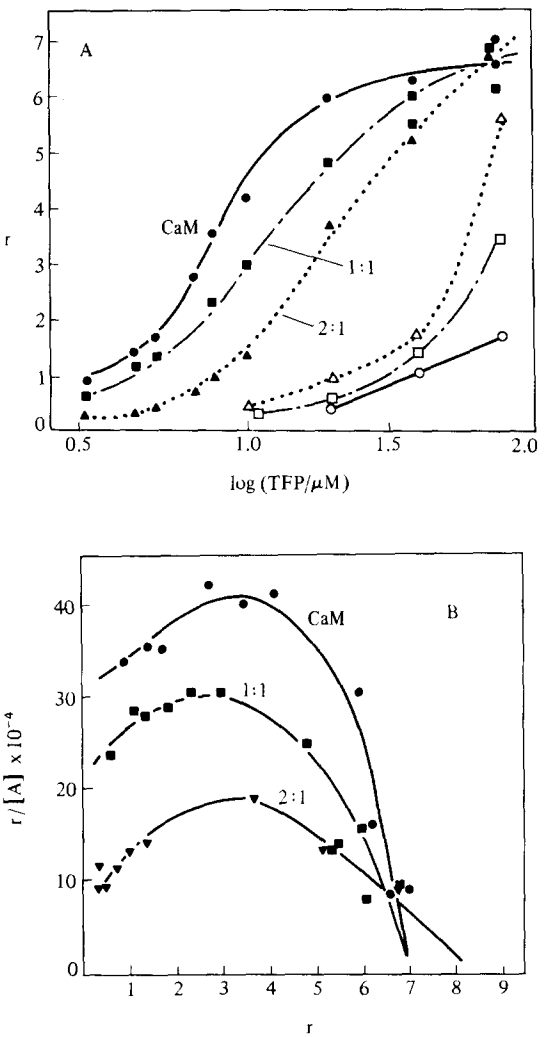

Fig. 3. Binding profiles for the association of trifluoperazine to calmodulin (●, ○), the 1:1 adduct (■, □) and the 2:1 adduct (▲, △). The closed symbols indicate the presence of $CaCl_2$ (1 mM) and the open symbols indicate the same in the presence of EGTA (2 mM). Panel A shows log concentration versus **r**, which is the binding ratio (moles of ligand bound per mole protein). Panel B shows the Scatchard plot of these data.

Table 1. Inability of the calmodulin 1:1 adduct to stimulate cyclic nucleotide phosphodiesterase activity

Calmodulin (nM)	% Activity*	1:1 Adduct (nM)	% Activity*
0.63	6 ± 4	16.2	3 ± 3
10.0	75 ± 7	260	9 ± 3
40.2	89 ± 5	4150	7 ± 3

* Basal activity is defined as 0%, and maximal activity with saturating calmodulin and calcium is 100%.

calmodulin, and the amount of this binding increased with covalent substitution with hydrophobic labeling reagent (Fig. 3A).

We have shown that the 2:1 adduct has no ability to stimulate phosphodiesterase activity [18]. The highly purified 1:1 adduct also exhibited no activity in the phosphodiesterase assay even at high concentrations relative to calmodulin (Table 1). Thus, under the conditions used, the 1:1 adduct had less than 0.01% of the activity of calmodulin.

Two nonreactive analogs (**III** and **IV**) of the labeling reagent were also assessed for binding to calmodulin. Figure 4 shows the binding obtained for **III**. The binding was greatly reduced relative to the binding found for trifluoperazine, and appeared to be Ca^{2+}-dependent. Solubility limited the amount of data obtained for this analog and the Scatchard plot proved incomplete. The plot did, however, suggest that N was considerably reduced relative to calmodulin (results not shown). Binding studies with **IV** yielded more complete data (Fig. 5). A Scatchard plot (inset, Fig. 5) suggests N = 2 with one class of binding sites for the binding to calmodulin. The 1:1 adduct exhibited binding which was additively reduced relative to calmodulin (N = 1). The binding experiment could not be quantitated for the 2:1 adduct since, above 60 μM trifluoperazine, no baseline could be established between the peak and the trough. Qualitatively, however, low levels of binding were detected.

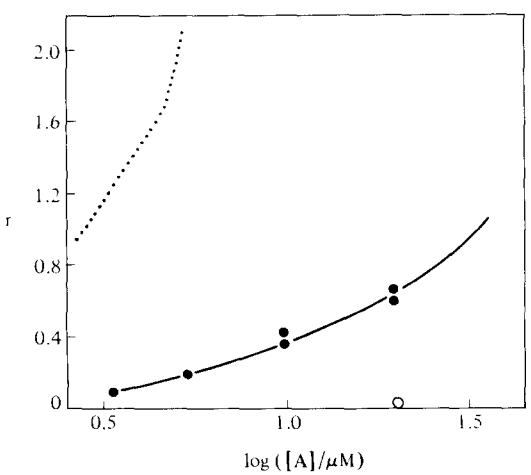

Fig. 4. Binding of **III** to calmodulin in the presence of Ca^{2+} (●) and the same with excess EGTA (○). These binding sites included the presence of 2% dimethyl sulfoxide (DMSO), but otherwise were done as in Fig. 3. The dotted line represents a part of the trifluoperazine binding curve taken from Fig. 3.

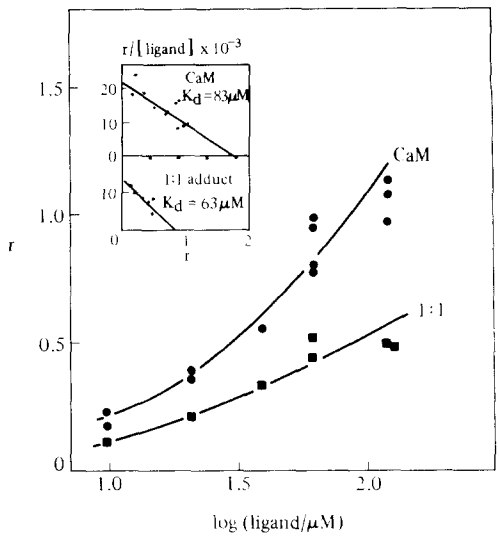

Fig. 5. Binding of **IV** to calmodulin (●) and the 1:1 adduct (■) in the presence of Ca^{2+}. The insets show the Scatchard plots of these data. These binding experiments included 2% DMSO, but otherwise were done as in Fig. 3.

DISCUSSION

Our results show that trifluoperazine binds to calmodulin with apparent positive cooperativity and a stoichiometry of about 7 moles/mole protein in the presence of Ca^{2+}. This finding is in agreement with two independent studies of calmodulin binding of another phenothiazine, chlorpromazine, in which stoichiometries of 5–7 were reported under conditions similar to those used herein [9, 10]. In addition, recent calorimetric studies showed that the total enthalpy change associated with trifluoperazine binding to calmodulin increased with increasing molar ratios of phenothiazine to calmodulin until a plateau occurred at a molar ratio of 7:1 [20]. In contrast, an earlier report suggested that trifluoperazine binds to calmodulin at only two sites in a Ca^{2+}-dependent manner with an apparent K_d of about 1 μM [8]. Under our conditions the binding midpoint was 8 μM. The reasons for the sizable discrepancy in the binding parameters of trifluoperazine to calmodulin are not known; however, the ionic strength used by us (225 mM) was much higher than that used by others (~10 mM, cf. Ref. 8). Also, the other study was performed over a much more limited concentration of trifluoperazine, and, thus, the additional sites could have been missed. Likewise, NMR has been used to monitor trifluoperazine binding to calmodulin [21–24], but, again, relatively limited concentrations of trifluoperazine were used. Also, there is no assurance of a linear relationship between signal intensity of protein spectral lines and

phenothiazine binding throughout a complete concentration range where binding occurs. The Hummel–Dreyer technique [19], on the other hand, is a more direct measure of bound material and not subject to the inherent assumptions and limitations associated with the spectral methods.

Our original data suggested that the labeling of calmodulin with **I** was affinity-mediated and that the covalent attachment of two hydrophobic probes inactivated calmodulin as a stimulator of cyclic nucleotide phosphodiesterase [18]. It is now clear that covalent attachment at Lys 75 with only one molecule of **I** is sufficient to inactivate calmodulin in this assay system. Inactivation by affinity labeling at one site was also observed by Newton *et al.* [25]. These calmodulin modifications argue that interaction at only one site is sufficient to interfere with enzyme interactions. The use of two other related compounds, **III** and **IV**, showed that stoichiometries of low magnitude do occur for the binding of hydrophobic substances to calmodulin. Conceivably, then, there may exist a limited number of more defined binding sites on calmodulin for hydrophobic compounds, and these sites may also interfere with calmodulin–enzyme interactions.

An explanation of the relatively high stoichiometries of chlorpromazine and trifluoperazine binding may lie in the amphipathic properties of these compounds. The measured critical micellar concentration (CMC) for chlorpromazine is 3 mM [26], much too high to directly affect the chlorpromazine–calmodulin studies discussed above. The CMC determination is, of course, done in the absence of protein. The presence of a limited number of hydrophobic sites on calmodulin may lead to the association of a large number of trifluoperazine (or chlorpromazine) molecules, due in large part to trifluoperazine–rifluoperazine interactions. These and other higher order interactions may also occur in solution well below the CMC.

The covalent attachment of one to two hydrophobic probes does not additively reduce trifluoperazine binding. If, as proposed above, more defined hydrophobic sites on the surface of calmodulin serve to nucleate a less specific interaction which leads to the association of many trifluoperazine molecules, then these less specific interactions would not be as strictly constrained as a "binding site" should be. The covalent attachment of the hydrophobic probe, **I**, does alter the interaction of trifluoperazine with calmodulin, reducing the affinity of the interaction. The EGTA form (i.e. Ca^{2+}-free form) of calmodulin binds only a minimal amount of trifluoperazine. The adducts bind much more trifluoperazine in the absence of Ca^{2+} (relative to calmodulin), but with low affinity. We feel that this observation is consistent with the proposal that some hydrophobic surface, in this case the attached reagent, can nucleate extensive trifluoperazine binding.

Compound **IV**, an analog of **I**, shows both more limited binding (N = 2) and an additive reduction in binding when one covalent probe is attached to calmodulin. Thus, one class of hydrophobic compounds, including felodipine [27], does bind to a limited number of sites on calmodulin. One of these two sites appears to be vicinal to Lys 75, since this residue is the site of covalent attachment of **I** on the adduct which binds only one molecule of **IV**. This site is consistent with the X-ray crystallographic localization of one molecule of trifluoperazine apparent in the difference density map after trifluoperazine has been diffused into calmodulin crystals (William J. Cook, personal communication). The aromatic portion of the trifluoperazine molecule was found to interact with the amino terminal globular domain (refer to Ref. 5 for crystal structure) between helices II and III with the piperazine ring extending towards the central helix that divides the protein into two globular domains. Lys 75 is on the proper face of this helix and is proximate to this hydrophilic extension of trifluoperazine.

It is interesting that Lys 77, located on the opposite face of the long helix (residues 65–92) connecting the two globular lobes of calmodulin [5], exhibits a vastly different reactivity than Lys 75 under Ca^{2+}-saturating conditions [28]. For example, Lys 75 and Lys 94 are the most reactive lysines in the protein, and Lys 77 has a lower than average reactivity. Yet, trifluoperazine at a stoichiometry of 2.5 moles/mole of calmodulin perturbs lysines 75, 77 and 148, with little effect on the other four lysines. β-Endorphin perturbs primarily lysines 75 and 148 [28], and β-endorphin 13-31 binds in a Ca^{2+}-dependent manner witi an apparent stoichiometry of 4–5 moles/mole of calmodulin [29]. Thus, calmodulin is capable of binding peptides and small hydrophobic compounds with stoichiometries ranging from 2 to 7 moles/mole of calmodulin. Although there appears to be some commonality in the binding sites [10], the exact nature of the molecular parameters that determine the binding characteristics have not been elucidated.

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REFERENCES

1. W. Y. Cheung, *Science* **207**, 19 (1980).
2. C. B. Klee and T. C. Vanaman, *Adv. Protein Chem.* **35**, 213 (1982).
3. L. J. Van Eldik, J. G. Zendegui, D. R. Marshak and D. M. Watterson, *Int. Rev. Cytol.* **77**, 1 (1982).
4. A. R. Means and J. G. Chafouleas, *A. Rev. Physiol.* **44**, 667 (1982).
5. Y. S. Babu, J. S. Sack, T. J. Greenhough, C. E. Bugg, A. R. Means and W. J. Cook, *Nature, Lond.* **315**, 37 (1985).
6. O. Herzberg and M. N. G. James, *Nature, Lond.* **313**, 653 (1985).
7. M. Sundaralingan, R. Bergstrom, G. Strasburg, S. T. Rao, P. Roychowdhury, M. Greaser and B. C. Wang, *Science* **227**, 945 (1985).
8. R. M. Levin and B. Weiss, *Molec. Pharmac.* **13**, 690 (1976).
9. D. R. Marshak, T. J. Lucas and D. M. Watterson, *Biochemistry* **24**, 144 (1985).
10. D. P. Giedroc, T. M. Keravis, J. V. Staros, N. Ling, J. N. Wells and D. Puett, *Biochemistry* **24**, 1203 (1985).
11. M. Sellinger-Barnette and B. Weiss, *Molec. Pharmac.* **21**, 86 (1982).

12. D. A. Malencik and S. R. Anderson, *Biochemistry* **21**, 3480 (1982).
13. D. P. Giedroc, D. Puett, N. Ling and J. V. Staros, *J. biol. Chem.* **258**, 16 (1983).
14. J. W. Taylor, R. J. Miller and E. T. Kaiser, *J. biol. Chem.* **258**, 4464 (1983).
15. D. P. Giedroc, N. Ling and D. Puett, *Biochemistry* **22**, 5584 (1983).
16. Y. Maulet and J. A. Cox, *Biochemistry* **22**, 5680 (1983).
17. J. A. Cox, M. Comte, J. E. Fitton and W. F. DeGrado, *J. biol. Chem.* **260**, 2527 (1985).
18. A. E. Jackson and D. Puett, *J. biol. Chem.* **259**, 14985 (1984).
19. J. P. Hummel and W. J. Dreyer, *Biochim. biophys. Acta* **63**, 530 (1962).
20. M. Tanokura and K. Yamada, *J. biol. Chem.* **260**, 8680 (1985).
21. S. Forsen, E. Thulin, T. Drakenberg, J. Krebs and K. Seamon, *Fedn Eur. Biochem. Soc. Lett.* **117**, 189 (1980).
22. R. E. Klevit, B. A. Levine and R. J. P. Williams, *Fedn Eur. Biochem. Soc. Lett.* **123**, 25 (1981).
23. J. Krebs and E. Carafoli, *Eur. J. Biochem.* **124**, 619 (1982)
24. T. Shimizu, M. Hatano, Y. Muto and Y. Nozawa, *Fedn Eur. Biochem. Soc. Lett.* **166**, 373 (1984).
25. D. L. Newton, T. R. Burke, Jr., K. C. Rice and C. B. Klee, *Biochemistry* **22**, 5472 (1983).
26. M. J. Conrad and S. J. Singer, *Biochemistry* **20**, 808 (1981).
27. J. S. Mills, B. L. Bailey and J. D. Johnson, *Biochemistry* **24**, 4897 (1985).
28. D. P. Giedroc, S. K. Sinha, K. Brew and D. Puett, *J. biol. Chem.* **260**, 13406 (1985).
29. D. P. Giedroc and D. Puett, *Molec. Pharmac.* **28**, 588 (1985).