Localization of a Trifluoperazine Binding Site on Troponin C†

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ABSTRACT: Trifluoperazine (TFP) was shown to interact with the cyanogen bromide fragment 9 (CB9) (residues 84–135) of rabbit skeletal troponin C and with a synthetic peptide representing the N-terminal region of CB9. The phenothiazine did not affect the calcium binding property of CB9 as observed by proton magnetic resonance and circular dichroism spectroscopies. The calculated calcium binding constants for CB9 in the presence and absence of trifluoperazine were identical ($K_{Ca^{2+}} = 1.3 \times 10^5$ M^{-1}). Localization of the trifluoperazine binding site was achieved by analyzing the ^1H NMR spectrum of CB9 and of a synthetic fragment corresponding to residues 90–104 of CB9. Drug-induced shifting and broadening of the ring protons of phenylalanine residues and the methyl resonances of alanine, leucine, and isoleucine residues suggest that the segment 95–102 is in close proximity to the phenothiazine aromatic region. The neighboring negative side chains in the peptide sequence also suggest that the single positive charge present on the piperazine nitrogens of trifluoperazine may interact with them and sterically block a region of interaction of calmodulin (CaM) and troponin C (TnC) with modulated proteins such as phosphodiesterase. Primary sequence analysis of CaM and troponin C reveals that a homologous hydrophobic region to site 3 is also found in the N-terminal region of site 1 of both calcium binding proteins. Binding of TFP to CB9 occurs both in the presence and absence of calcium since the hydrophobic region in these small fragments is completely accessible to TFP whether calcium is present or not. The dissociation constant of the drug to apoCB9 (8 μM) was obtained by ellipticity measurements at 222 nm and was comparable to the 5 μM value obtained by Levin and Weiss [Levin, R. M., & Weiss, B. (1978) *Biochim. Biophys. Acta 540*, 197–204] for calcium-saturated rabbit skeletal troponin C.

The importance of calcium as a second messenger has been demonstrated by the discovery of numerous regulatory calcium binding proteins. Small acidic proteins like troponin C and parvalbumin in muscle tissues, S-100 in brain tissues, and the ubiquitous protein calmodulin are all examples of the troponin C superfamily of calcium-regulated proteins (Barker et al., 1978). A substantial sequence homology exists between these proteins in the regions coordinating the calcium ions.

In particular, troponin C and calmodulin possess four calcium binding sites per molecule (Potter & Gergely, 1975; Klee et al., 1980). However, the metal-binding affinity of these sites varies significantly ($K_{Ca^{2+}}$ values ranging from 10^5 to 10^7 M^{-1}). These two proteins thus exibit high and low calcium-affinity sites. The high-affinity sites of CaM[1] are sites 1 and 2 while sites 3 and 4 in troponin C are the high-affinity sites (Potter & Gergely, 1975; Kilhoffer et al., 1980a,b; Wang et al., 1982). In addition, it has been well documented that calmodulin can replace TnC in releasing the inhibitory effect of troponin I on the skeletal muscle actomyosin ATPase. Reciprocally, troponin C can activate the Ca^{2+}/calmodulin-regulated rat testis phosphodiesterase (Dedman et al., 1977). Note that in both cases, the biological activity of these hybrid complexes was lower than the one observed for the natural complexes. Thus it is evident that both proteins share similar functional binding regions to troponin I (TnI) and rat testis phosphodiesterase.

It was shown by Levin & Weiss (1977) that the activation of bovine brain phosphodiesterase by calmodulin could be selectively inhibited by phenothiazine antipsychotics. The specificity of these drugs for calmodulin was partly demonstrated by the failure of other calcium binding proteins to bind trifluoperazine. Besides calmodulin, only troponin C displayed a significant calcium-dependent binding of trifluoperazine (Levin & Weiss, 1978). Recently however, the brain-specific calcium binding protein S-100 has been shown to interact in a calcium-dependent manner with a phenothiazine-bound Sepharose affinity column (Marshak et al., 1981). Two high-affinity calcium dependent trifluoperazine binding sites ($K_{diss} = 1$ μM) exist on calmodulin. At concentrations of TFP above 10 μM, troponin C binding of this drug was comparable to calmodulin binding ($K_{diss} = 5$ μM) (Levin & Weiss, 1978). The number of calcium-dependent TFP binding sites on troponin C has not been established.

Controversy about the correlation between drug binding to calmodulin and their actual pharmacological potency has arisen from the demonstration that pharmacologically inactive isomers of these drugs can still inactivate a calmodulin-regulated phosphodiesterase assay (Norman et al., 1979; Roufogalis, 1981). The interaction of these drugs with calmodulin has thus been qualified as a nonstereospecific hydrophobic one. The overall fact still remains that a hydrophobic region on calmodulin is exposed upon Ca^{2+} binding (LaPorte et al., 1980) and this region is involved in the binding of calmodulin to receptor molecules such as phosphodiesterase, phosphorylase kinase, and troponin I (Dedman et al., 1977; Kuznicki et al., 1981). The presence of a hydrophobic domain was further

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[1] Abbreviations: Ac(A^{98})STnC(90–104)amide, synthetic N-terminal acetylated rabbit skeletal troponin C fragment, residues 90–104 with a C-terminal amide and alanine substituted at position 98; CaM, calmodulin; CB9, cyanogen bromide fragment 9, residues 84–135 of rabbit skeletal troponin C; CD, circular dichroism; Chelex 100, styrene lattice resin with iminodiacetic acid exchange groups; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-pressure liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; ^1H NMR, proton nuclear magnetic resonance; Hse, homoserine; Pipes, 1,4-piperazinediethanesulfonic acid; QAE, quaternary anion exchanger; R_f, relative mobility of a compound on TLC plates relative to the solvent front; $NaDodSO_4$, sodium dodecyl sulfate; TFP, trifluoperazine; TLC, thin-layer chromatography; TnC, calcium binding unit of skeletal muscle troponin; TnI, inhibitory subunit of skeletal muscle troponin.

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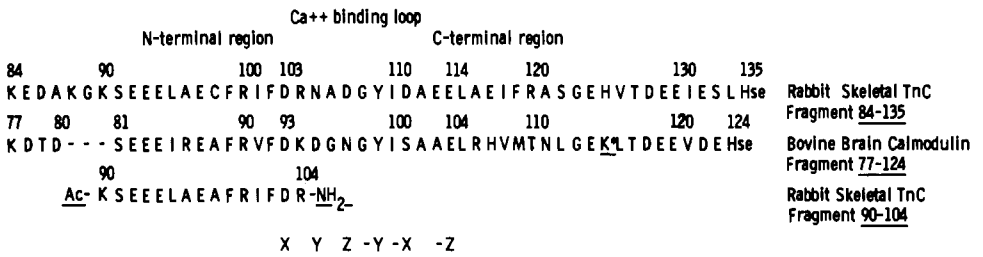

FIGURE 1: Primary sequence alignment of peptides shown to bind phenothiazines. Fragment 84–135 of rabbit skeletal troponin C is abbreviated CB9 in this study. Fragment 77–124 of bovine brain calmodulin was recently shown to bind to a fluphenazine affinity column in the presence of calcium (Head et al., 1982). Fragment 90–104 represents our synthetic fragment comprising the proposed TFP binding site. The letters X, Y, Z, –Y, –X, and –Z represent the calcium-coordinating ligands of the peptides. The symbol K* stands for trimethyllysine. The deletion between residues 80 and 81 in the calmodulin fragment sequence represents the alignment optimization described by Barker et al. (1978).

proven by recent CaM purification schemes (Charbonneau & Cormier, 1979; Jamieson & Vanaman, 1979; Gopalakrishna & Anderson, 1982).

Recent ^1H NMR and ^{113}Cd NMR studies were performed on whole calmodulin in order to investigate the influence of trifluoperazine on the overall structure of the modulator and to gain some insight on where the drug might bind (Forsén et al., 1980; Klevit et al., 1981; Krebs & Carafoli, 1982). Although regions of binding of trifluoperazine have been postulated to be close to methionine and phenylalanine residues in CaM, all authors mentioned have used a drug concentration range in which the drug itself undergoes aggregation as shown in the present study. Thus caution should be used in assessing the drug behavior above the 1 mM range and the interpretation of interactions between trifluoperazine and protein.

Our group has been interested in identifying a particular trifluoperazine binding site on troponin C. Both troponin C and calmodulin have been shown to possess a similar site of interaction on phosphodiesterase and troponin I while phenothiazines appear to block the phosphodiesterase activity by binding to a calcium-exposed hydrophobic region of calmodulin. Recent CD work by our group[2] using synthetic fragments of site 3 of rabbit skeletal troponin C has led us to the conclusion that phenothiazines bind to the N terminal of this high-affinity calcium binding site and induce helix formation upon binding. Our goal in this paper is to describe the location of this trifluoperazine binding site on the N terminal of site 3 of rabbit skeletal troponin C by using native and synthetic fragments, the sequences of which are shown in Figure 1.

Experimental Procedures

Materials. Protected amino acid derivatives and others reagents used for the synthesis were described elsewhere (Reid et al., 1981). DTNB was purchased from Sigma (St. Louis, MO). Trifluoperazine was a gift from Smith Kline and French (Mississauga, Canada) and was used without further purification.

Preparation of Ac(A^{98})STnC(90–104)amide. The peptide was synthesized by solid-phase peptide synthesis on a Beckman 990 peptide synthesizer. A benzhydrylamine resin support was used to produce a C-terminal amide group. The amino acid protection strategy and the peptide cleavage approach were as described in an earlier paper (Reid et al., 1981). The crude peptide was initially purified on a QAE A-25 column (30 cm × 1 cm) with a 100 mM KH_2PO_4/10 mM KCl, pH 6.50 buffer as the eluent. The major peak from this purification was lyophilized and desalted either on a Bio-Gel P-2 column equilibrated in 50 mM NH_4HCO_3 or by reverse-phase high-pressure liquid chromatography on a SynChropak RP-R C_{18} preparative column (1 cm × 25 cm) (Linden, IN) with a 0.1% F_3CCOOH/water, 0.1% F_3CCOOH/acetonitrile gradient. For this later purification method, a 0–60% gradient of 0.1% F_3CCOOH/acetonitrile (60-min gradient with a flow rate of 2 mL/min) was sufficient to elute the peptide as a single peak. The composition and concentration of the pure peptide were verified by amino acid analysis (Table I).

Table I: Amino Acid Analysis of CB9 Fragment and Ac(A^{98})STnC(90–104)amide

	CB9 fragment		Ac(A^{98})-STnC(90–104)amide	
amino acid	obsd	sequence	obsd	sequence
Asp	6.29	6	0.98	1
Thr	1.03	1		
Ser	2.54[a]	3	0.78[a]	1
Glu	11.80	12	3.94	4
Gly	3.20	3		
Ala	5.80	6	1.99	2
Val	1.16	1		
Ile	3.77	4	1.00	1
Leu	3.07	3	1.02	1
Tyr	0.96	1		
Phe	3.03	3	2.03	2
Lys	3.17	3	1.05	1
His	0.92	1		
Arg	3.35	3	2.19	2
Hse	1.40	1		
Cys	nd[b]	1		

[a] Serine was partly destroyed during peptide hydrolysis. [b] nd stands for not determined.

Preparation of Cyanogen Bromide Fragment 9 of Rabbit Skeletal Troponin C. Rabbit skeletal troponin C was prepared as described earlier (Chong & Hodges, 1981). This protein was then modified with DTNB in order to protect cysteine-98 from modification during the cyanogen bromide cleavage (Nagy et al., 1978). After dialysis of the excess DTNB and lyophilization of the material, the troponin C was treated with a 200-fold excess of cyanogen bromide in 70% formic acid. The reaction was left to proceed for 24 h. Digestion was complete as demonstrated by the absence of a troponin C band on $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of the digest. This digest was fractionated on a Sephadex G-50 superfine column (190 cm × 1.5 cm) equilibrated in 50 mM NH_4HCO_3. The CB9-containing peak was further purified on the same column. This material showed a single band on $NaDodSO_4$–urea–polyacrylamide gel electrophoresis and a single peak by reverse-phase high-pressure liquid chromatography. The HPLC column used in this case was the preparative C_{18} SynChropak RP-P column mentioned above. The peptide CB9 was eluted from the column in a 0–60% gradient of 0.1% F_3CCOOH/acetonitrile (60-min gradient with a flow rate of 2 mL/min). The peptide sequence and concentration were determined by amino acid analysis (Table I).

[2] R. E. Reid, J. Gariépy, and R. S. Hodges, unpublished results.

Trifluoperazine Solutions. Trifluoperazine was obtained as a hydrochloride powder. The purity of this material was assessed by thin-layer chromatography on silica gel plates (Merck). The first solvent system used was composed of 3 g of ammonium acetate in 20 mL of water and 100 mL of methanol. The R_f value of the only spot observed under both UV light and phenothiazine staining conditions (see below) was 0.40. The second system used was composed of ammonium hydroxide, benzene, and dioxane at a ratio of 5:60:35, respectively. The R_f value for the drug was 0.32. The positive identification of trifluoperazine was obtained by the sulfuric acid/formaldehyde test described by Clarke (1969). The concentration of a typical TFP solution prepared was determined both by weight and by optical density measurement at 256 nm, using an extinction coefficient value of 30 110 M^{-1} cm^{-1} (Clarke, 1969). Both approaches yielded similar values. Because of the light sensitivity of phenothiazines, only fresh solutions of the drug were prepared and kept in the dark for the remainder of the experiment. Typically, 5–30 mM stock solutions were made up for the CD and NMR experiments. Note that the pH of these stocks solutions was low (2.8–3.3), depending on the trifluoperazine hydrochloride concentration. We avoided going above a drug concentration of 1 mM since at these concentrations, the drug outbuffered the buffering capacity of our NMR samples. The pH of the sample at the end of the titration was reduced by only 0.05 of a pH unit under these conditions. Other reasons motivated our decision not to exceed this 1 mM TFP range and are described under results.

Preparation of CD Samples. The CB9 sample was dissolved in 100 mM Mes, 50 mM KCl, 1 mM DTT, and 1 mM EGTA, pH 6.0, buffer to a concentration of 0.4 mM. The rabbit skeletal troponin C was dialyzed against a 50 mM NH_4HCO_3, 1 mM EGTA, and 1 mM DTT solution, followed by water in the presence of Chelex 100 resin. The lyophilized sample was dissolved in 100 mM Mes, 50 mM KCl, and 1 mM DTT, pH 6.0, buffer to a concentration of 1 mg/mL. The concentration of these samples was verified by amino acid analysis.

Preparation of ^1H NMR Samples. The peptide CB9 was dissolved in 15 mM Pipes, 0.1 M KCl, and 0.2 mM DSS, pH 6.80. A 2-μL aliquot of β-mercaptoethanol was added to the 400-μL NMR samples before the experiment. As observed in a preliminary NMR spectrum, the addition of a reducing agent is essential for the proper folding of the fragment (spectrum not shown). As for the peptide Ac(A^{98})STnC-(90–104)amide, it was taken up in 25 mM KH_2PO_4/50 mM KCl, pH 6.0. The concentration of each peptide sample is stated in each figure legend.

Circular Dichroism Experiments. CD spectra were obtained on a Jasco J-500C spectropolarimeter. Typically, a 700-μL sample solution was placed in a 0.05-cm cell. The ellipticity values obtained at 222 nm for the calcium titrations were used to evaluated the calcium binding constant of CB9. The nonlinear fitting procedure used was described elsewhere (Reid et al., 1981). In the case of the TFP titration of CB9 in the absence of calcium, the binding constant of the drug was evaluated from the following Hill plot equation:

$$\log [Y/(1 - Y)] = \log K_{assoc} + m \log [TFP]_{free}$$

where Y is the ratio of observed ellipticity at 222 nm over the maximum ellipticity recorded. K_{assoc} represents the association constant of TFP with peptide CB9, and $[TFP]_{free}$ represents the calculated concentration of the free drug during each step of the titration. A linear least-squares fitting of the data gave a correlation coefficient of 0.99 while the observed slope was 1.4.

Proton NMR Experiments. Proton NMR experiments were performed on a Bruker HXS 270-MHz spectrometer operating in a Fourier-transform mode and equipped for quadrature detection. For the peptide Ac(A^{98})STnC(90–104)amide, a typical spectrum was obtained from 1000 acquisitions under a 1-s acquisition time with a 9-μs pulse width and a line broadening of 1 Hz. For the CB9 fragment, the spectrum was obtained from 4000 acquisitions under a 0.5-s acquisition time. All other parameters were as described above.

Metal Ion Analysis. The calcium solutions used in this study were prepared from reagent-grade $CaCl_2 \cdot 2H_2O$ dissolved in either 15 mM Pipes/0.1 M KCl, pH 6.80, for the NMR experiments or 100 mM Mes/50 mM KCl, pH 6.00, for the CD experiments. The calcium content was determined by EDTA titration with murexide as the end point indicator (Blaedel & Knight, 1954). Calcium was removed from the peptide CB9 and rabbit skeletal troponin C by dialysis against EGTA or by treatment of the sample with Chelex 100 resin.

Results

Trifluoperazine Behavior in Solution. The two nitrogen atoms of the piperazine moiety of trifluoperazine have respective pK_as of 3.9 and 8.4 (Chatten & Harris, 1962). This implies that at the experimental and physiological pH range used (6.0–7.4), the molecule possesses a positive charge on one of the piperazine nitrogens. Going to low pHs will protonate both nitrogens and will affect some of the aliphatic ^1H NMR resonances (results not shown). Going to high pHs (higher than 7.5) will promote TFP precipitation due to the hydrophobic nature of trifluoperazine in its unprotonated form. This fact can be correlated with the observed sharp decrease in drug binding to CaM at pHs above 7.5 (Levin & Weiss, 1977).

Increasing the drug concentration at constant pH produces large shifts in the ^1H NMR spectrum of trifluoperazine (Figure 2). Changes in the observed chemical shifts of the drug resonances probably arise from the aggregation or stacking of the phenothiazine aromatic rings. Most of the protons of the drug appear shielded from or by the aromatic network in this "aggregated" geometry. Increasing the concentration of anionic buffers like phosphate (Figure 2B) may promote the drug interaction with itself by bridging together the positive charges of two piperazine groups, for example. This may explain the readily observed resonance of the piperazine ring methyl protons (2.7 ppm), which appears in a fast-exchange mode (sharp peak) in the presence of phosphate at a relatively low TFP concentration (0.25 mM). In contrast, we observe only a broad peak at 2.7 ppm, in the absence of phosphate for a 0.5 mM TFP solution. A related behavior of the drug in Pipes was noted. We suspect that other sulfonic acid type buffers like Mes and Mops may also produce such artifacts. We have thus performed all our experiments at TFP concentrations not exceeding 1 mM. A control spectrum of the drug alone was recorded for each addition of the drug during TFP titrations of the peptides[3] in buffers such that the ^1H NMR spectra remain unaffected by the changing concentration of trifluoperazine.

Addition of Calcium to CB9 in the Presence and Absence of TFP. In the absence of TFP and calcium, the CB9 ^1H NMR spectrum obtained corresponds to apoCB9 (Birnbaum & Sykes, 1978) and closely resembles the spectrum of synthetic fragments of this site (Gariépy et al., 1982) (Figure 3A). As

[3] Copies of the aliphatic regions of the calcium titrations of both CB9 and CB9 in the presence of TFP as well as control spectra for the drug titrations of CB9 are available upon request. Control spectra of the drug alone are identical with those shown in Figure 5B.

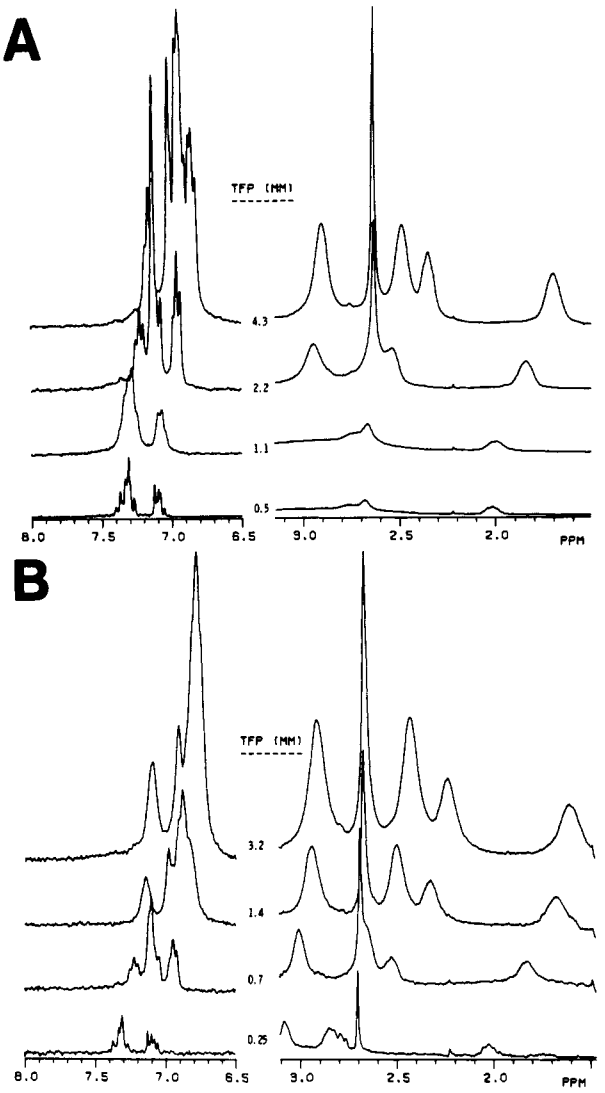

FIGURE 2: Effect of TFP and divalent anion concentration on ^1H NMR spectrum of TFP. (Panel A) No divalent anion present but increasing TFP concentration. The spectra were recorded in 50 mM KCl, pH 6.0. (Panel B) Phosphate present and increasing TFP concentration. The spectra were recorded in 300 mM KH_2PO_4/50 mM KCl, pH 6.0.

calcium was added, most of the spectral features expected were observed including the shift of tyrosine-109 ring proton resonances to 6.64 and 6.51 ppm and the appearance of upfield-shifted resonances in the 0.2–0.8-ppm region.[3] Shifts in this region are thought to arise from methyl groups of leucine or isoleucine side chains placed in proximity to aromatic side chains (Birnbaum & Sykes, 1978; Gariépy et al., 1982). Also the methyl group of one or more of the six alanines present in CB9 is affected by the presence of calcium as the resonance envelope situated around 1.4 ppm is altered considerably.[3]

In the presence of TFP (Figure 3B), the addition of calcium generates the same Ca^{2+}-induced resonances observed in the absence of drug [the tyrosine-109 resonances at 6.64 and 6.51 ppm (Figure 3A) as well as the alanine methyl resonances at 1.4 ppm[3]]. In general, the presence of the drug does not appear to alter the binding of calcium to the coordinating region of CB9 and is thus evidence that the drug binding site is probably not located in the calcium-coordinating region.

Addition of TFP to CB9 in the Presence and Absence of Calcium. Figure 4A illustrates the effect of TFP on CB9. In the absence of calcium, the addition of TFP affects both the phenylalanines and the drug aromatic region (7.0–7.4-ppm

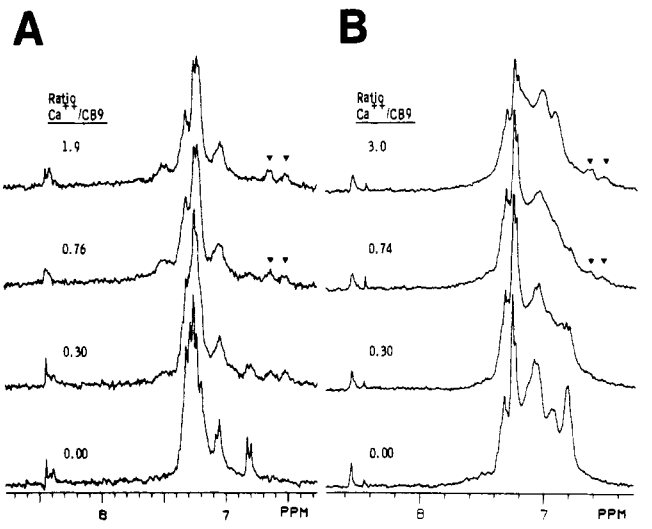

FIGURE 3: Calcium titration of CB9 in the presence and absence of TFP. (Panel A) Calcium titration of the aromatic region of CB9 in the absence of trifluoperazine. [CB9] = 0.33 mM. (Panel B) Calcium titration of the aromatic region of CB9 in the presence of trifluoperazine (0.37 mM). [CB9] = 0.34 mM. Experimental conditions are described under Experimental Procedures. Symbols highlight changes in the ^1H NMR spectrum.

region), when compared to the initial spectrum of CB9 (no drug) and the TFP control spectrum.[3] A large broadened multiplet appears at 6.9 ppm. Similarly, the leucine/isoleucine methyl region centered at 0.9 ppm and the alanine methyl region at around 1.4 ppm are significantly influenced by the presence of this phenothiazine. Thus it appears that a region of CB9 involving phenylalanine, alanine, and leucine and/or isoleucine side chains is in spacial proximity to the drug.

Trifluoperazine titration of this cyanogen bromide fragment in the presence of calcium is shown in Figure 4B. Some features arising from the presence of calcium are retained (tyrosine-109 ring-proton resonances for example), while changes occur in the phenylalanine envelope (around 7.25 ppm) with new aromatic resonances appearing as a broadened multiplet centered at 6.9 ppm. Changes are also observed in the aliphatic upfield-shifted resonances (0.2–0.8 ppm) and the methyl region of leucine and isoleucine (0.9-ppm area). These resonances are not observed in the drug control spectrum.[3]

Localization of Drug Binding Site. A 15-residue synthetic fragment of the N-terminal region of CB9 (lacking the Ca^{2+} binding loop) was synthesized in order to further elucidate the site of interaction. It appeared clear from earlier studies (Nagy et al., 1978; Reid et al., 1981; Gariépy et al., 1982) that residues 95–102 will adopt an α-helical conformation upon Ca^{2+} binding to CB9 and produce an hydrophobic patch composed of phenylalanine side chains in particular. Figure 5A shows the effect of adding TFP to the peptide. Again, large changes in both the phenylalanine-99 and -102 resonances (7.3 ppm) and the phenothiazine aromatic resonances (7.1 and 7.3 ppm) cannot be accounted for by the summation of the individual peptide and drug spectra (Figure 5B). The aliphatic region again shows shifting and broadening in the leucine/isoleucine methyl region (0.9 ppm) and the alanine-98 methyl resonance at 1.3 ppm (Gariépy et al., 1982). Thus the sequence involving Leu-95 to Phe-102 appears to interact with trifluoperazine.

Circular Dichroism Experiments. The effects of trifluoperazine on the secondary structure of CB9 can be monitored by circular dichroism. In the absence of calcium, CB9 already possesses some secondary structure (−5700 degrees of ellipticity at 222 nm). This was in accordance with the value of −5730

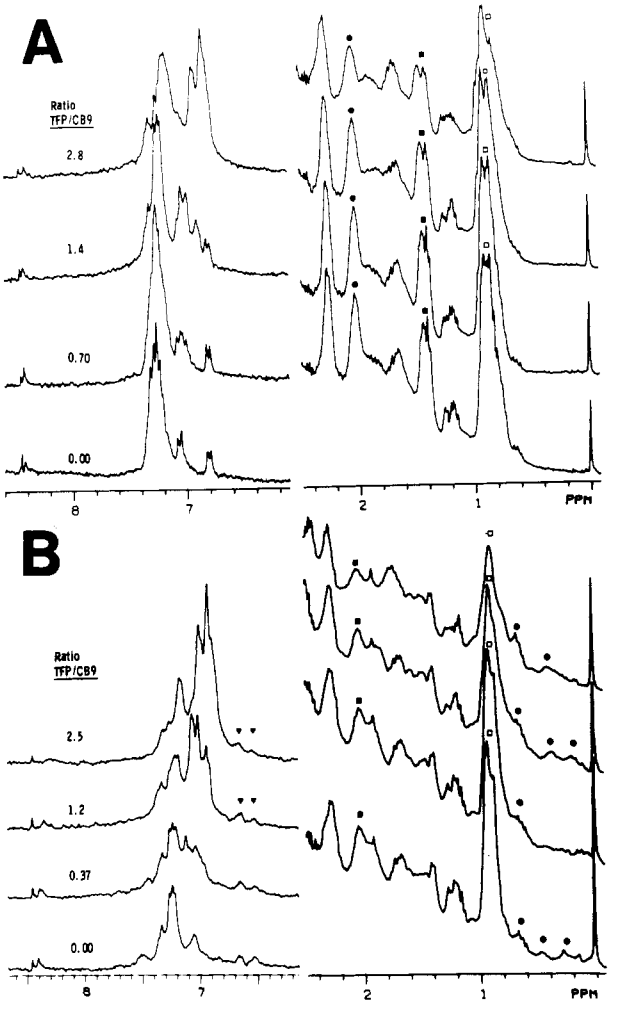

FIGURE 4: Trifluoperazine titration of CB9 in the presence and absence of calcium. (Panel A) TFP titration of CB9 in the absence of calcium. [CB9] = 0.27 mM. (Panel B) TFP titration of CB9 in the presence of calcium (2.6 mM). [CB9] = 0.33 mM. Experimental conditions are described under Experimental Procedures. Control spectra of TFP at addition of drug corresponding to the CB9/TFP titration were recorded and are available upon request.[3] At the concentrations of TFP used, no shifting of resonances in the control spectra was observed. Symbols highlight changes in the ^{1}H NMR spectrum.

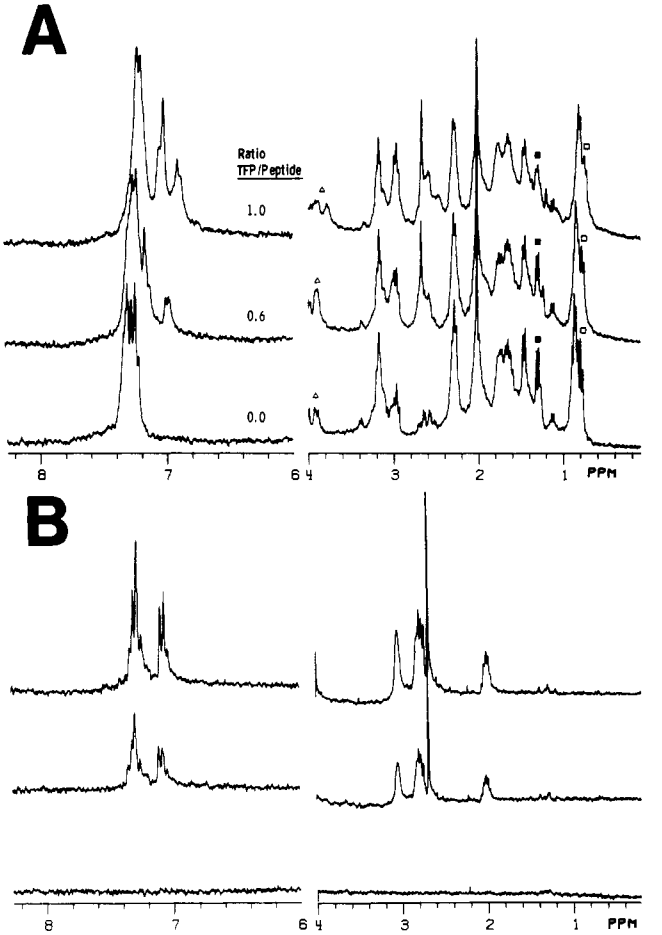

FIGURE 5: Trifluoperazine titration of synthetic fragment Ac(A^{98})-STnC(90–104)amide. The peptide concentration was 1.0 mM. Experimental conditions are described under Experimental Procedures. (Panel A) ^{1}H NMR spectrum of the fragment at various additions of the drug. (Panel B) Control spectrum of trifluoperazine at additions of drug corresponding to the TFP/peptide titration. Symbols highlight changes in the ^{1}H NMR spectrum.

observed by Nagy et al. (1978). Figure 6 indicates that the addition of drug to a TFP/peptide ratio of 1.1:1 induces a further increase in helical content (spectrum B). We did not go beyond a phenothiazine concentration of 1 mM because of a significant noise factor introduced in the CD spectrum by TFP. In the presence of calcium, the maximum ellipticity content of CB9 is induced so that addition of TFP yielded no observable effect on the CD spectrum.

Calcium titration in the presence or absence of trifluoperazine yielded identical calcium binding constants of 1.3×10^5 M^{-1}, in agreement with $K_{Ca^{2+}}$ values of 2.6×10^5 M^{-1} for a synthetic 34-residue fragment of this region (Reid et al., 1981) and 5×10^5 M^{-1} for an earlier study of CB9 (Nagy et al., 1978).

Determination of the drug binding constant in the absence of calcium was possible with CB9 from the ellipticity change observed at 222 nm. The binding constant calculated was equal to 1.2×10^5 M^{-1} (K_{diss} = 8 μM), which is comparable to the observed dissociation constant of 5 μM obtained from equilibrium dialysis experiments on calcium-saturated rabbit skeletal troponin C (Levin & Weiss, 1978). Thus it appears that the flexibility of this fragment allows for the induced folding of the site by TFP and unlike the native protein, does not necessitate the binding of calcium to induce the proper TFP binding site geometry.

The trifluoperazine titration of rabbit skeletal troponin C was monitored by circular dichroism. The results showed no increase in ellipticity at 222 nm upon TFP addition in the presence or absence of calcium.

Discussion

Binding of phenothiazines to CaM may yield information about the mode of action of this class of drugs. Even if the binding of such antipsychotics has been qualified as arising from a nonstereospecific hydrophobic interaction (Norman et al., 1979; Roufogalis, 1981), their inhibition of CaM-regulated phosphodiesterase activity suggests that other CaM-regulated functions may be altered in a similar fashion. It thus appeared of interest to identify the drug binding sites on CaM.

Our decision to investigate a particular region of troponin C was based on the following earlier observations: (1) Fragments containing regions of bovine brain CaM (78–148 and 1–106) as well as the cyanogen bromide fragment CB9 of rabbit skeletal troponin C were shown to interact with rabbit skeletal TnI and release the inhibition of actomyosin ATPase by troponin I (Weeks & Perry, 1978; Grabarek et al., 1981; Kuznicki et al., 1981). It was further proven that a homologous 11–12-residue region of troponin C (89–100) and of CaM (78–90) is an important region in the binding of TnI to these calcium binding proteins (Grabarek et al., 1981; Kuznicki

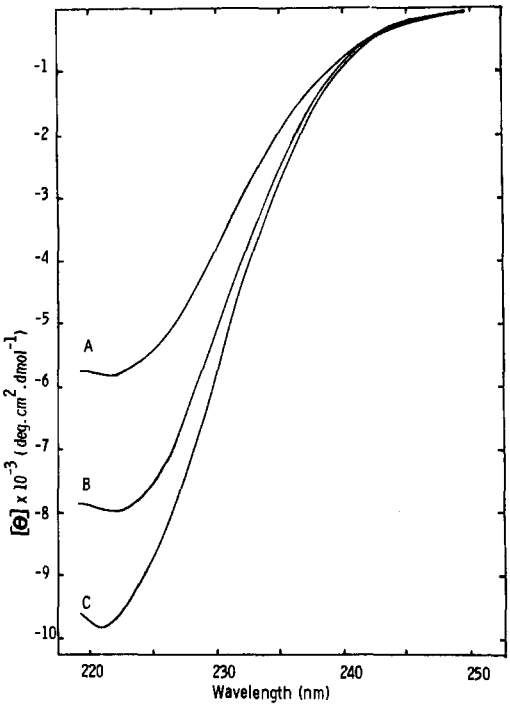

FIGURE 6: Circular dichroism results for peptide CB9. [CB9] = 0.6 mM. (Spectrum A) CB9 spectrum in the absence of calcium and trifluoperazine. (Spectrum B) CB9 spectrum in the absence of calcium but in the presence of trifluoperazine, TFP/CB9 ratio of 1.6. (Spectrum C) CB9 spectrum in the presence of excess calcium with or without trifluoperazine, TFP/Ca^{2+}/CB9 ratios of 1.6:8:1 and 0:8:1.

et al., 1981). (2) A cyanogen bromide fragment of bovine brain calmodulin spanning the region 77–124 was shown to bind in the presence of calcium to a fluphenazine–Sepharose column (Head et al., 1982). This region, as illustrated in Figure 1, largely corresponds to the CB9 sequence of rabbit skeletal troponin C (residues 84–135). Similarly, trifluoperazine has been shown to interact with the TR2C fragment of CaM (residues 78–148) as demonstrated by its inhibition of the TR2C-stimulated phosphorylase kinase activity (Kuznicki et al., 1981). (3) Finally, we have recently observed by circular dichroism the binding of TFP and the induction of helix in a synthetic 34-residue peptide (residues 90–123) of rabbit skeletal troponin C.[2]

Thus the peptide region of troponin C comprising the sequence 90–100 appeared to be a trifluoperazine binding region. Our first step was to make use of a cyanogen bromide fragment of troponin C containing both the proposed region of TFP binding and also a high-affinity calcium binding site. This peptide abbreviated CB9 contains residues 84–135 (Figure 1).

The presence or absence of calcium did not appear to affect the interaction of trifluoperazine with CB9. In both cases, the ^1H NMR results revealed large chemical shifts in the aromatic regions of CB9 and TFP, which could not be accounted for by the summation of the peptide and drug spectra (Figure 4). Changes were also observed in the 0.9-ppm region of the spectra, which corresponds to methyl-proton resonances of leucine and isoleucine residues. The fact that calcium was not a necessary requirement for drug binding was confirmed by circular dichroism results that showed an increase in ellipticity at 222 nm upon addition of TFP to apoCB9 (Figure 6). The calculated drug binding constant was 1.2×10^5 M^{-1} in the absence of calcium. Since, upon calcium addition, no more ellipticity at 222 nm could be induced, it was impossible to determine by this approach the phenothiazine binding constant to CB9 in the presence of calcium. Levin & Weiss (1978) estimated the dissociation constant of the drug to troponin C to be 5 μM in the presence of calcium, which closely corresponds to the CB9 dissociation constant in the absence of calcium ($1/K_{assoc}$ = 8 μM) determined in this study.

Though the binding of phenothiazines to both CaM and TnC has been shown to be Ca^{2+} dependent (Levin & Weiss, 1978), the finding that TFP binds to apoCB9 is not surprising. There is no evidence that these drugs do not bind to isolated single Ca^{2+} binding sites represented by CB9 (residues 84–135 of TnC) and by the region 77–124 of calmodulin, in the absence of calcium. This binding of the drug to CB9 in the absence of calcium can be explained by the fact that the N-terminal region of CB9 is probably flexible and exposed in the fragment and readily induced to the correct conformation by the drug. Thus binding does not require addition of calcium to induce and expose the correct conformation. Support for this conclusion comes from the CD results where the N terminal of CB9 is induced by both calcium and TFP.

Proton magnetic resonance and circular dichroism results have demonstrated that the presence of the drug does not affect the binding of calcium to CB9. Equivalent binding constants of 1.3×10^5 M^{-1} were calculated from the ellipticity measurements at 222 nm in the presence or absence of trifluoperazine. Similarly, characteristic resonances at 6.64 and 6.51 ppm for the tyrosine ring protons can be observed in the calcium-saturated spectrum of CB9 with or without TFP being present (Figures 3A & 3B).

It appears that the side chains of phenylalanines and methyl groups of leucine and/or isoleucine are in spacial proximity to the drug. We strongly suspected that the region 90–104 was the site of trifluoperazine binding. This fragment was synthesized and used to test this hypothesis. We did not expect a strong TFP binding to the peptide since the fragment lacks the actual calcium binding site and appears to possess little secondary structure as monitored by circular dichroism. Drug addition did not yield any significant CD change, probably due to a weaker TFP binding constant to such a fragment. Some CD constraints also made this approach inadequate to study the TFP/peptide interaction. For example, the upper limit of 1 mM TFP and the necessity to have a high concentration of a small peptide to observe a few residues of helix being formed do not permit us to go far enough in the titration. However, the proton NMR results pointed out again large shifts in the phenylalanine resonances around 7.5 ppm, and the aliphatic alanine (1.4 ppm), leucine, and isoleucine methyl resonances (1.0-ppm region) were affected by the presence of the phenothiazine (Figure 5A). These altered resonances correspond to those observed in CB9 and suggest that the amino acid sequence 95–102 of CB9 interacts with TFP. Nagy et al. (1978) commented that the N-terminal helical region of CB9 containing residues 94–103 is induced by Ca^{2+} and stabilized by hydrophobic patches formed by residues 95, 98, 99, 101, and 102. This statement was recently confirmed by our work on synthetic peptides of that site (Reid et al., 1981; Gariépy et al., 1982).

Evidence to support our belief that the TFP-specific binding site on CaM involves a short helical hydrophobic sequence comes from the reported calcium-induced exposure of a hydrophobic surface on CaM. LaPorte et al. (1980) reported that such a domain interacts with CaM-regulated proteins (i.e., phosphodiesterase) and also with hydrophobic ligands like trifluoperazine. Furthermore, the presence of this particular site is made use of in the purification of CaM by hydrophobic affinity chromatography (Charbonneau & Cormier, 1979; Jamieson & Vanaman, 1979; Gopalakrishna & Anderson,

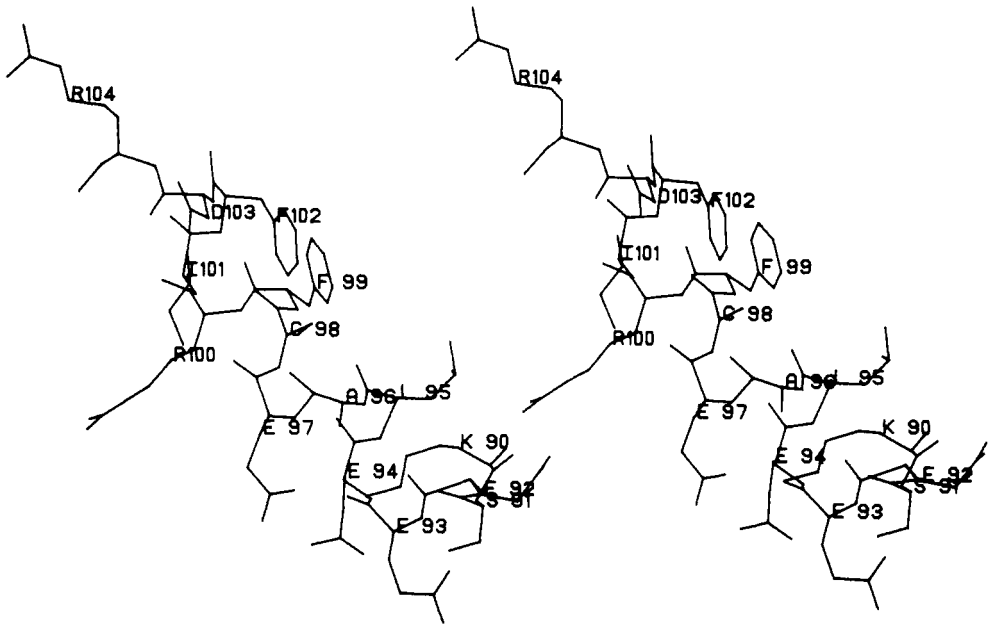

FIGURE 7: Stereoprojection of rabbit skeletal troponin C region 90–104. This representation was constructed from the crystal coordinates of carp parvalbumin for the N-terminal region of the CD hand (residues 38–52) (Kretsinger & Nockolds, 1973). The original set of coordinates for the peptide backbone was retained, but the rabbit skeletal troponin C side chains of residues 90–104 were substituted to the parvalbumin ones. The orientation of the side chains along their respective C^{α}–C^{β} bond was maintained. This drawing can more easily be viewed in stereo with a stereoscope obtained from Hubbard Scientific Company, Northbrook, IL.

1982). Using such a purification approach, Head et al. (1982) have shown that a fluphenazine affinity column retains, in the presence of calcium, a cyanogen bromide fragment of CaM (77–124) homologous to CB9 of troponin C (Figure 1).

In view of the present results, we suggest that the drug acts as a steric blocker of a hydrophobic interphase, in proximity to, or part of the binding site to phosphodiesterase in CaM and to TnI in both TnC and CaM. This hydrophobic site is probably a small helical region involving a stretch of 7 or 8 amino acids rich in aliphatic and aromatic side chains resembling the sequence 95–102 of rabbit skeletal troponin C. A key feature of this segment is the presence of two phenylalanine rings separated by one turn of helix, which orients both aromatic side chains on the same side of the helix.

Another interesting feature that may favor the binding of trifluoperazine is the presence of a positive charge at physiological pH on the piperazine part of the molecule. Evidence from circular dichroism results on a synthetic peptide representing the region 90–123 of rabbit skeletal troponin C has indicated a correlation between the distance separating the aromatic and positively charged domains of phenothiazines and their ability to increase the α-helical content of this analogue upon drug binding. Promethazine and chlorpromazine, for example, possess short positively charged aliphatic chains and only weakly induce the formation of α helix while trifluoperazine and fluphenazine both have a long piperazine moiety and yield a large increase in ellipticity at 222 nm upon binding to the peptide.[2] Also, the addition of TFP to CB9 in the presence or absence of calcium promotes the broadening of the β-CH_2 and γ-CH_2 resonances of glutamic acid side chains (2.1- and 2.3-ppm region; Figure 4), a result that suggests a reduction in mobility of some of these side chains of CB9. In the case of trifluoperazine, the positive charge of the drug is situated 6–10 Å away from its aromatic region depending on which of the piperazine nitrogens is carrying the positive charge. The presence of this flexible "piperazine arm" of TFP may thus be crucial in the blocking of negatively charged side chains of troponin C and CaM thought to be involved in an interaction with TnI (Grabarek et al., 1981) (residues such as Glu-92, -93, -94, and -97). It may well explain the differential ability of trifluoperazine to inactivate the CaM-regulated phosphodiesterase activity over the less potent phenothiazine chlorpromazine (Levin & Weiss, 1979).

A stereoprojection of the proposed TFP binding site is presented in Figure 7. This projection was constructed from the peptide backbone coordinates of carp parvalbumin segment 38–52 (Kretsinger & Nockolds, 1973). The representation points out the proximity of the two phenylalanine rings. Rotation along the phenylalanine C^{α}–C^{β} bond can bring the aromatic side chains even closer. These rings may thus lie along the same plane and permit the stacking of the TFP rings, or the drug aromatic system may intercalate between the phenylalanine side chains. As for the piperazine positive charge, it remains close to most of the glutamic acid side chains if one considers the flexibility of both the negatively charged side chains and the piperazine arm.

The interaction of trifluoperazine and other phenothiazines with calmodulin and TnC can be correlated with the fact that these drugs offer structural features present in peptides and proteins shown to interact with CaM and TnC. The primary sequence alignment of several opioid peptides and of CaM binding proteins has indicated the presence of eight residue long sequences containing both a hydrophobic and a positively charged domain (Malencik & Anderson, 1982; Sellinger-Barnette & Weiss, 1982).

Levin & Weiss (1977) mentioned the fact that two TFP high-affinity sites exist on CaM in the presence of Ca^{2+}. Phosphorylase kinase activation assays on calmodulin fragments (Kuznicki et al., 1981) pinpointed that TFP binding sites exist on a fragment containing the calcium binding sites 1 and 2 and on another fragment containing sites 3 and 4, the first fragment, however, having a stronger affinity for TFP. A search through the primary sequence of CaM and TnC was performed in order to find possible helical sites having two phenylalanines separated by one turn of helix. Figure 8 shows four possible regions. The N-terminal regions of sites 1 and 3 of both proteins are homologous for this short helical se-

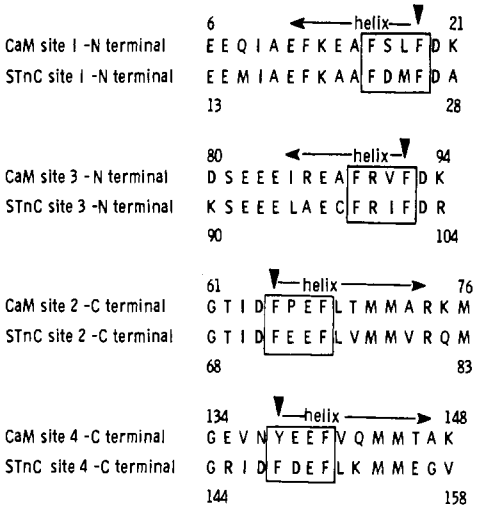

FIGURE 8: Homologous sequence regions of rabbit skeletal troponin C and bovine brain CaM containing short helical regions involving two aromatic side chains separated by one turn of helix. Framed sequences represent possible sites of hydrophobic interaction with the aromatic moiety of trifluoperazine. Arrows indicate the beginning and direction of helical regions.

quence while the beginnings of the C terminal of sites 2 and 4 are also quite similar and offer two other possibilities. Some evidence suggests that the N-terminal region of site 1 might be the other TFP high-affinity site. Rabbit skeletal troponin C has ten methionines, but upon addition of 4 mol of calcium, methionine-25 is preferentially labeled by the hydrophobic fluorescent tag dansylaziridine (Johnson et al., 1978). This methionine is part of the hydrophobic segment of site 1 (Figure 8). This result suggests that this segment is selectively exposed in the presence of calcium. The C terminal of site 4 is a less probable choice since the CaM tyrosine-138 ring proton resonances are not affected by the drug presence (Klevit et al., 1981; Krebs & Carafoli, 1982). It is thus doubtful that the drug reaches this part of the molecule (Figure 8). The oxidation of CaM methionines by chlorosuccinimide in the presence of calcium suggested that methionine-71, -72, -76, and possibly-109 were exposed in the Ca^{2+}-saturated state (Walsh et al., 1978). This modified calmodulin showed a reduced ability to activate a CaM-dependent phosphodiesterase. Klevit et al. (1981) and Krebs & Carafoli (1982) have demonstrated by ^1H NMR the shifting of methionine resonances of CaM in the presence of TFP to suggest that one of the drug binding sites was located near these methionines and thus our proposed site-2 region. However, caution should be observed in making such an assignment since Walsh et al. (1978) mentioned that the modified CaM secondary structure had been altered significantly by the chemical treatment and the loss of regulatory activity may not be solely due to the oxidation of these particular amino acids.

In conclusion, this work has pointed out that the region 92–102 of rabbit skeletal troponin C and probably 82–92 of bovine brain calmodulin, by homology, offer hydrophobic and negatively charged regions favoring the binding of trifluoperazine. A homologous segment in site 1 of both CaM (8–19) and TnC (15–26) appears to offer a similar trifluoperazine binding site.

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Registry No. Ac(A^{98})STnC(90–104)amide, 84433-41-0; TFP, 117-89-5.

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Stopped-Flow Studies of Myelin Basic Protein Association with Phospholipid Vesicles and Subsequent Vesicle Aggregation†

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ABSTRACT: When mixed with vesicles containing acidic phospholipids, myelin basic protein causes vesicle aggregation. The kinetics of this vesicle cross-linking by myelin basic protein was investigated by using stopped-flow light scattering. The process was highly cooperative, requiring about 20 protein molecules per vesicle to produce a measurable aggregation rate and about 35 protein molecules per vesicle to produce the maximum rate. The maximum aggregation rate constant approached the theoretical vesicle–vesicle collisional rate constant. Vesicle aggregation was second order in vesicle concentration and was much slower than protein–vesicle interaction. The highest myelin basic protein concentration used here did not inhibit vesicle aggregation, indicating that vesicle cross-linking occurred through protein–protein interactions. In contrast, poly(L-lysine)-induced vesicle aggregation was easily inhibited by increasing peptide concentrations, indicating that it did cross-link vesicles as a peptide monomer. The myelin basic protein:vesicle stoichiometry required for aggregation and the low affinity for protein dimerization suggested that multiple protein cross-links were needed to form a stable aggregate. Stopped-flow fluorescence was used to estimate the kinetics of myelin basic protein–vesicle binding. The half-times obtained suggested a rate constant that approached the theoretical protein–vesicle collisional rate constant.

Myelin protects and insulates selected nerve axons from the surrounding environment. Myelin membranes contain approximately 27% protein of which 31% is myelin basic protein (Rumsby & Crang, 1977). Myelin basic protein is rich in lysine and arginine residues and is believed to interact electrostatically with negatively charged phospholipids (Boggs & Moscarello, 1978a). Nonionic interactions have also been reported (Stollery et al., 1980; Boggs et al., 1981). Most evidence indicates that the protein is found only on the cytoplasmic side of myelin membranes (Peterson & Gruener, 1978; Golds & Braun, 1976; Poduslo & Braun, 1975).

One proposed role for myelin basic protein is maintenance of the multilaminar structure of the myelin sheath through membrane cross-linking. Chemical cross-linking of myelin basic protein in solution and in myelin indicated that the protein may span the intracellular space between adjacent lamellae as a dimer (Golds & Braun, 1978a,b). Self-association studies have also shown that myelin basic protein dimerizes in solution (Smith, 1980) and in the presence of detergent monomers (Smith, 1982). However, other work indicated that the protein was isolated as a monomer at high detergent concentrations and as a dimer at low detergent concentrations (Smith & McDonald, 1979). In addition, results of solubilization studies with the two fragments generated by BNPS-skatole[1] treatment of the protein led to the identification of the C terminal as the site for ionic interaction with lipids (Jones & Rumsby, 1977). These results were viewed as support for lamellae cross-linking as a protein monomer (Rumsby & Crang, 1977). Other studies indicated that both the N- and C-terminal peptides, generated by BNPS-skatole, interact with lipids (London et al., 1973; Boggs et al., 1981). Myelin basic protein was found to have a folded structure (Epand et al., 1974) which may be important in forming the membrane binding region.

Myelin basic protein has been reported to aggregate phospholipid vesicles (Smith, 1977a,b; Boggs & Moscarello, 1978b; Lampe & Nelsestuen, 1982). Aggregation of vesicles may constitute a model for cross-linking of myelin membranes. However, the turbid mixture that results from vesicle aggregation makes many simple physical measurements difficult. These problems can be minimized by analysis of changes immediately following mixing when the protein and vesicles are largely monomeric. The following study used stopped-flow fluorescence and light scattering techniques to study the association of myelin basic protein with phospholipid vesicles and the initial rates of vesicle aggregation. The kinetic parameters obtained can be used to calculate thermodynamic and stoi-

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[1] Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; dansyl-PE, *N*-dansylphosphatidylethanolamine; HPLC, high-performance liquid chromatography; *n*, sample size; *r*, correlation coefficient; BNPS-skatole, 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]indolenine.

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