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A Theoretical Study of the Binding of Phenothiazine Derivatives to Residues 82–93 of Calmodulin

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SUMMARY

A theoretical study was performed of the interaction of four phenothiazine derivatives, promethazine, promazine, trifluopromazine, and trifluoperazine, with a fragment (82–93) of calmodulin, held in the α-helical conformation. The computations were performed in the framework of the SIBFA 2 procedure (sum of interactions between fragments computed *ab initio*), which uses analytical formulas based on *ab initio* self-consistent field computations. The interaction energy is the sum of the intermolecular phenothiazine-oligopeptide interaction energy and of the separate intramolecular energy variations of the phenothiazine and of the side chains of the oligopeptide upon complex formation. The ordering of interaction energies of the four investigated phenothiazines parallels the ordering of their experimentally measured affinities for calmodulin, with a maximum affinity for trifluoperazine. The principal features of the trifluoperazine complex are a short hydrogen bond between the piperazinium proton and one anionic oxygen of Glu 87, and hydrophobic interactions between the piperazinium ring and Val 91 and between the methylene chain and Ala 88, together with partial insertion of the phenothiazine ring and the —CF_3 substituent between Phe 89 and Phe 92.

CaM is a Ca^{2+}-binding protein, discovered in 1970 (1, 2), which regulates a considerable number of Ca^{2+}-based enzymatic processes and is actively involved in cell motility, cell growth, cell transformation, etc. (reviewed in Refs. 3–6). Following the challenging demonstration that phenothiazine binding to CaM inhibits the CaM-dependent activation of cyclic nucleotide phosphodiesterase (7, 8), an accumulating body of evidence indicates that phenothiazines and anti-CaM drugs can interfere with a number of CaM-dependent metabolic processes, such as DNA repair (9–11), microtubule assembly (12), cell proliferation (13), and tumor cell growth (14–16), to name a few.

Evidence for the existence of two major phenothiazine-binding sites in CaM was provided in Ref. 8. In the present study, we undertake a search, by means of theoretical computations, for the intrinsically preferred binding modes of a series of four related phenothiazine derivatives, to the fragment (82–93) of CaM (see Fig. 1). This study was stimulated by the convergence of three recent groups of reports, as follows.

1) Demonstration, by mean of ^1H NMR, that the binding of trifluoperazine to a fragment (84–135) of the closely related Ca^{2+}-binding protein, TnC, occurs in fragment 90–104 (17), and that this fragment is induced into an α-helical conformation (17–19). The amino acid sequence of this oligopeptide fragment is highly conserved in the corresponding sequence (80–94) of CaM. It comprises glutamate residues in the N-terminal part and a patch of hydrophobic residues, the proton resonances of which are chemically broadened and shifted upon trifluoperazine binding (17). The adoption of an α-helical conformation by sequence 82–92 of CaM is itself supported by the recent resolution of its crystal structure (20).

2) The availability of detailed structure-activity relationships among phenothiazine derivatives (21–25), outlining the requirements for improved CaM binding, the principal of which are a separation of at least three methylene groups between the cationic end and the phenothiazine ring and appropriate substitutions in the −2-, −3-, or −4-positions of the latter.

3) The availability of an explicit proposal, based on CPK model building, for the structure of the complex of anti-CaM drugs with the fragment (82–93) of CaM (26, 27). According to this proposal, the cationic end of a phenothiazine drug, such as trifluoperazine, is in close proximity to Glu 84 and Glu 87, whereas the methylene chain and the polycyclic aromatic ring should be involved in hydrophobic interactions with residues Ile 85, Ala 88, Phe 89, and Phe 92. We will use this specific model as a starting point for our own computations and will try to assess, on a series of four representative phenothiazine derivatives, namely, PMZ, PZ, TFPZ, and TFP (see Fig. 2), to what extent the reported evolution of their binding affinities for CaM (21–25) can be accounted for *quantitatively* by theoretical computations. Upon phenothiazine binding, the α-helical conformation of the backbone is retained. This assumption is borne out by the above mentioned circular dichroism results

ABBREVIATIONS: CaM, calmodulin; TnC, troponin C; PMZ, promethazine; PZ, promazine; TFPZ, trifluopromazine; TFP, trifluoperazine; SIBFA, sum of interactions between fragments computed *ab initio*; PCILO, perturbative configuration interaction using localized orbitals.

- Glu_{82}-Glu_{83}-Glu_{84}-Ile_{85}-Arg_{86}-Glu_{87}-Ala_{88}-Phe_{89}-Arg_{90}-Val_{91}-Phe_{92}-Asp_{93} -

Fig. 1. Sequence of the fragment (82–93) of calmodulin.

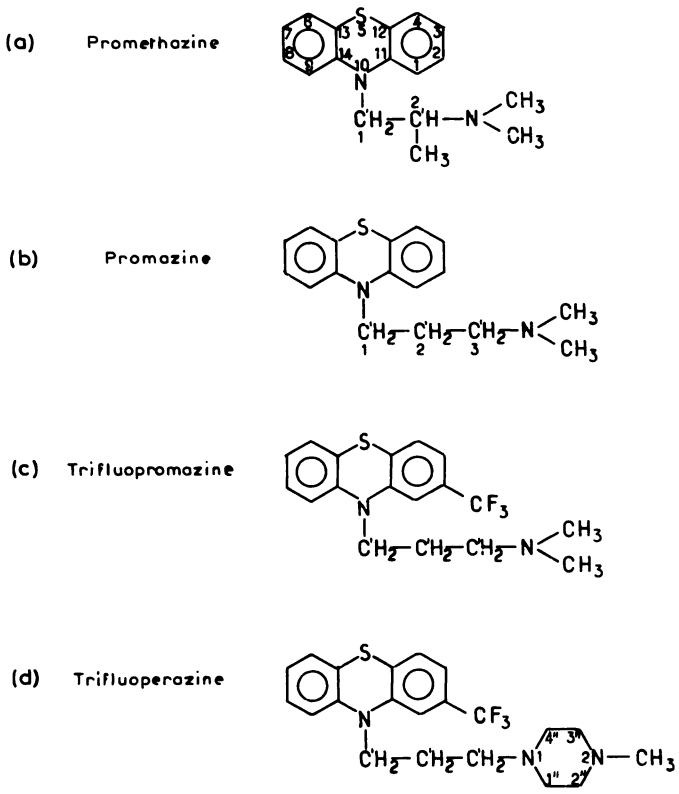


Fig. 2. The phenothiazines investigated in this study. Structural formulas and atom numbering are shown for: (a) promethazine, (b) promazine, (c) trifluopromazine, and (d) trifluoperazine.

of Refs. 17 and 18, devoted to a cyanogen bromide fragment of the closely related TnC, which are indicative of an enhanced α-helical content upon trifluoperazine binding. Together with the intermolecular drug-oligopeptide interaction energy, we will optimize by means of energy minimization the conformational energy of the interacting phenothiazine and that of the oligopeptide side chains, by performing the appropriate conformational changes.

Procedure

Intramolecular interactions. The variations of the intramolecular interaction (conformational) energies of the oligopeptide and of the phenothiazine upon complex formation were computed in the framework of the SIBFA procedure (28, 29). Within this methodology, both interacting entities are built of elementary constitutive fragments separated by single bonds, and the variation of intramolecular energy upon a conformational change is obtained as the variable part of the sum of the interactions between the fragments expressed as:

$$\delta E = E_{MTP} + E_{pol} + E_{rep} + E_{disp} + E_{tor} \quad (1)$$

In this expression, E_{MTP} and E_{pol} are the electrostatic and polarization contributions, computed using a multipolar expansion of the *ab initio* self-consistent field procedure molecular wave functions of the fragment; E_{rep} and E_{disp} are the repulsion and dispersion contributions, respectively; and E_{tor} is a torsional contribution, calibrated (28) for elementary rotations along C—C and C—O bonds.

Intermolecular interactions. The intermolecular drug-oligopeptide interaction energies were accordingly computed by the SIBFA 2 procedure (30) as a sum of five terms:

$$\Delta E = E_{MTP} + E_{pol} + E_{rep} + E_{disp} + E_{CT} \quad (2)$$

in which E_{CT} is a charge-transfer contribution, the definition of the first four contributions being the same as in Expression 1.

The fragments utilized to construct the phenothiazine derivatives were the following: the N-methyl phenothiazine ring and a trifluomethyl group; for PMZ, PZ and TFPZ, one methane and a trimethylammonium group; for TFP, one methane and N_1-methyl, N_2-methyl piperazinium. Standard bond lengths were adopted at the junctions between two fragments. In PMZ, the cationic and aromatic moieties are directly linked together, the methane substituting the cationic moiety, whereas in PZ, TFPZ, and TFP it is interposed between the cationic and aromatic moieties. To allow for conformational changes along the N_{10}—C_1 and C′—N bonds and computation of the corresponding energy change, both the aromatic and the cationic moieties are themselves subsequently split into two subfragments (e.g., phenothiazine and methane; methane and dimethylammonium) with the multipolar expansion of the original C—N bonds being shared equally by two superimposed fictitious junctional bonds, the barycenters of which are located so as to coincide with that of the original bond. The piperazinium proton was located on N_1 rather than on N_2, that is, at the same distance from the phenothiazine ring as in PZ or TFPZ. Examination of a molecular model in the light of the proposals of Reid (26) readily indicates that this location should allow for a better interaction than on N_2. Furthermore, anti-CaM drugs lacking the N_2 nitrogen (that is, with a piperidine ring rather than a piperazine ring), such as the phenylbutylpiperidines or the butyrophenones, are endowed with CaM affinities comparable to or superior to that of TFP (23), an indication in favor of a preferential location of the proton on N_1 rather than on N_2.

Standard bond lengths and valence angles were adopted throughout (31). The internal geometry of the phenothiazine ring was taken from Ref. 32. The *ab initio* self-consistent field procedure computations on the constitutive fragments were performed using our usual basis set (33). In the computations of the intramolecular energy of the oligopeptide, the value of E_{MTP} was simplified to that of the sole monopole-monopole component, which enabled a considerable reduction of the total computing time. The validity of this assumption for the oligopeptide was preliminarily assessed and is justified by the fact that the conformational behavior of the oligopeptide upon phenothiazine binding is dominated by the repulsive interactions between the glutamate residues Glu 84 and Glu 87, in which the monopole-monopole term is, by and large, the dominating part of E_{MTP}. Thus, in the PMZ, TFPZ, and TFP complexes, the values of the Glu 84-Glu 87 interaction energies δE are 67.0, 67.0, and 63.9 kcal/mol, respectively. The energies in the PMZ and TFPZ complexes are thus higher, by an amount of 3.1 kcal/mol, than in the TFP complex. This difference is close to the corresponding differences of δE_{cal} values, with respect to the TFP complex, of 2.4 and 2.7 kcal/mol, respectively. The numerical values of δE are, furthermore, close to the corresponding values of E_{MTP} of 68.7, 68.7, and 65.3 kcal/mol in the respective PMZ, TFPZ, and TFP complexes, themselves very close to the corresponding values of the monopole-monopole contributions of 69.7, 69.7, and 66.0 kcal/mol, respectively.

The search for the optimal configuration of the complex was performed by energy minimization (34) of the sum of δE_{cal} (oligopeptide) *plus* δE_{phen} (phenothiazine) *plus* ΔE (phenothiazine-oligopeptide). The involved variables in the minimization process are the six variables defining the position of the drug with respect to the oligopeptide, the four torsional angles of the drug, and the torsional angles of the amino acid side chains which are involved in the binding and, more particularly, Glu 84, Glu 87, Phe 89, and Phe 92.

The computations were performed on the CRAY-1 supercomputer of the Centre de Calcul Vectoriel pour la Recherche using a vectorized version of the SIBFA program.

Results and Discussion

Preferred Conformations of CaM (82–93) Side Chains and of the Phenothiazines Prior to Complexation

Oligopeptide. Two complexes involving amino acid side chains of opposite signs were optimized at both ends of the oligopeptide. The first one, between Glu 82 and Arg 86, involves the two carboxylate oxygens and the imino and vicinal amino guanidinium protons. The second one, between Asp 93 and Arg 90, involves one carboxylate oxygen and the imino and vicinal amino guanidinium protons.

Glu 83, which is too far from the assumed binding site to be involved in a specific intermolecular interaction with the phenothiazines, was held in an *all-trans* conformation so as to minimize the electrostatic repulsions with the neighboring glutamate residues. Glu 84 and Glu 87 (which will be involved in the binding), were found to adopt at the issue of optimization, *trans-trans* and *trans-gauche* conformations, respectively, along their C_α—C_β and their C_β—C_α bonds, in order to minimize their mutual electrostatic repulsions. The optimized torsional angles χ_1 and χ_2 of both Phe 89 and Phe 92 are close to 270° and 90°, respectively. They are 300° and 300° for Ile 85, for torsions counted along the C_α—C_β and the C_β—C_γ bonds, respectively. Finally, the two methyl Val 91 groups are rotated 180° and 300° with respect to the backbone along the C_α—C_β bond. All methyl side chains are in a staggered conformation.

The total intramolecular energy of the oligopeptide in the so defined conformation, which includes the side chain-side chain and the side chain-backbone interactions, will be taken as energy zero. When complex formation takes place, the only side chain residues to undergo a conformational change are Glu 84, Glu 87, Phe 89, and Phe 92.

Phenothiazine derivatives. *PZ, TFPZ, TFP.* Conformational energy maps of the three phenothiazines with a three-methylene chain as a function of the torsional angles τ_1 (S—N—C′$_1$—C′$_2$), τ_2 (N—C′$_1$-C′$_2$—C′$_3$), for values of τ_3 (C′$_1$—C′$_2$—C′$_3$—C′$_4$), and τ_4 (C′$_2$—C′$_3$—C′$_4$—N) prefixed at 180° and 20° increments have been computed. They are reported in the Appendix. The global energy minimum corresponds to a *gauche-gauche* conformation with $\tau_1 = 40°$, $\tau_2 = -60°$, stabilized by the interaction between the cationic head and the electron-donating phenothiazine ring. Another *gauche-gauche* conformation ($\tau_1 = -40°$, $\tau_2 = 60°$) corresponds to a local energy minimum. The nonequivalence of the two minima is due to the fact that the geometry of the phenothiazine ring in the adopted crystal structure (32) is not symmetrical with respect to the S-N axis. A *gauche-trans* conformation ($\tau_1 = 40°$, $\tau_2 = 180°$) corresponds to a local energy minimum in the series PZ, TFPZ, and TFP. Its energy separation with respect to the global energy minimum amounts to 3.4 kcal/mol in PZ and 2.8 kcal/mol in TFPZ and TFP. Further refinement of the global energy minimum by performing energy minimization of the conformational energy, as a function of the four torsional angles τ_1, τ_2, τ_3, and τ_4 starting from $\tau_1 = 40°$, $\tau_2 = 300°$, resulted in a further deepening of the *gauche-gauche* to *gauche-trans* energy separation of about 3 kcal/mol. The energy-minimized values of τ_1, τ_2, τ_3, and τ_4 are for the three derivatives of 30°, 295°, 140°, and 170°, respectively. This conformation of the phenothiazines will be taken as the energy zero of Table 1.

Let us compare the conformational energy map of the PZ molecule with the PCILO map of Ref. 35 drawn with 30° increments and devoted to a representative phenothiazine derivative with a folding angle of 140°. (It is to be noted, however, that this compound has a tetramethylammonium cationic head instead of trimethylammonium.) The global energy minimum also corresponds to a *gauche-gauche* conformation, similar to the SIBFA result. The *gauche-trans* conformation, in contrast, is now of equivalent stability to *gauche-gauche*, instead of being a local minimum. Possible reasons for this difference of results are the larger delocalization of the cationic charge in the tetramethylammonium head than in the trimethylammonium head, resulting in a weakened electrostatic interaction in *gauche-gauche*, and/or a difference in the representation of the electrostatic contribution in PCILO which assumes a zero-differential overlap hypothesis. On the whole the SIBFA map is more restricted than the PCILO one, although the general shapes are similar.

The conformation of the phenothiazine derivatives in their crystal structure (see, e.g., Ref. 32 and references therein) generally corresponds to a *gauche-trans* conformation. Crystal packing forces as well as environmental effects at large are largely able to overcome the energy difference with respect to the global energy minimum as was reviewed in Ref. 36.

Promethazine. A conformational energy map of PMZ as a function of τ_1 and τ_2, using 20° increments with τ_3 prefixed at 180°, indicates that the global energy minimum is located at $\tau_1 = 40°$, $\tau_2 = 320°$ (see Appendix). A local energy minimum now occurs for a *gauche-trans* conformation defined by $\tau_1 = 320°$, $\tau_2 = 180°$, but the energy separation is large ($\delta = 11$ kcal/mol). Energy minimization as a function of τ_1, τ_2, and τ_3 starting from the position of the global minimum on the energy map resulted in values of τ_1, τ_2, and τ_3 equal to 35°, 295°, and 200°, respectively, for the intrinsically preferred conformation of PMZ the energy of which will be taken as energy zero.

We will investigate here the binding to the CaM (82–93) oligopeptide of the phenothiazines in their *gauche-trans* conformation, with the exception of PMZ, which will remain in its intrinsically preferred *gauche-trans* conformation (see below). This *gauche-trans* conformation allows for a simultaneous interaction of the cationic end with Glu 84 and Glu 87, on the one hand, and of the methylene chain and phenothiazine ring with Ile 85, Ala 88, Phe 89, and Phe 92 on the other hand, with the possible partial insertion of the phenothiazine ring between Phe 89 and Phe 92 (26). In this connection, we recall the conclusions reached in studies of environmental effects on the conformation of biomolecules (36). Thus, it was shown, for example, that whereas the monocationic form of histamine adopts *in vacuo* a highly folded conformation (37), solvation results in an extension of the structure, resulting in a coexistence of *trans* and *gauche* conformations.

The Oligopeptide (82–93) Phenothiazine Complexes

As mentioned in the introduction, we have undertaken a search, by means of energy minimization, for specific oligopeptide-phenothiazine complexes that would be stabilized by the simultaneous occurrence of both ionic and hydrophobic interactions. The coordinates of the complexes at the outcome of optimization are available upon request. The stereochemistries of the optimized complexes will be detailed below. The PMZ, TFPZ, and TFP complexes are represented in Fig. 3 (a–c). For the sake of clarity, only the hydrogens belonging to the phenothiazines are represented.

The most conspicuous conformational changes of the oligopeptide which occur upon phenothiazine binding are the ones

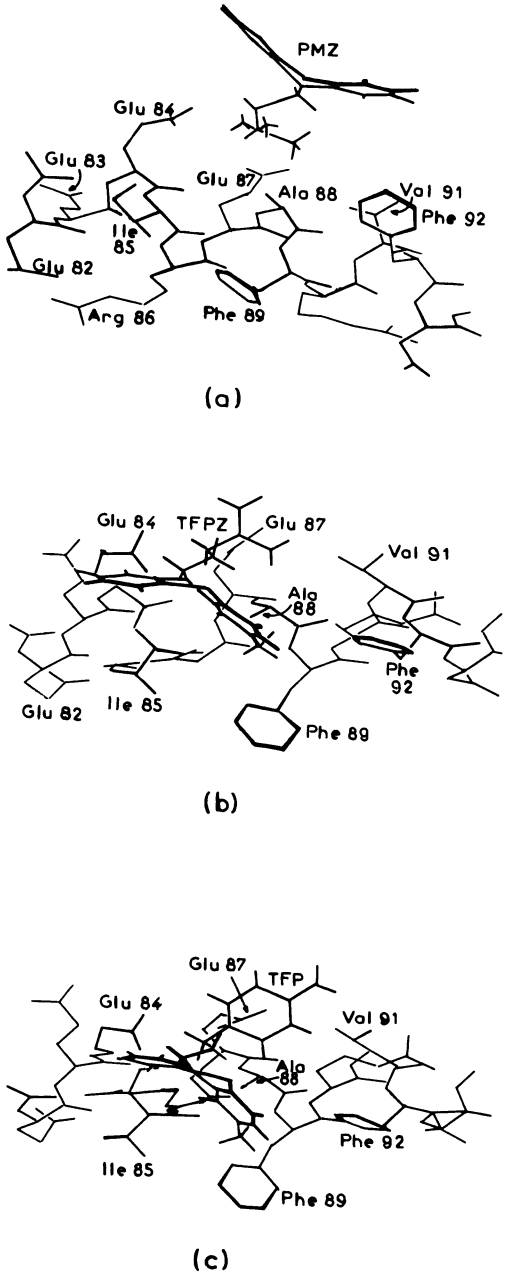


Fig. 3. Stereochemistry of the CaM oligopeptide complexes with (a) PMZ, (b) TFPZ, and (c) TFP.

involving Glu 84 and Glu 87, which are brought closer together by the attraction of the phenothiazine cationic end. Thus, the values of χ_1, χ_2, and χ_3 of Glu 84 vary from 180°, 175°, and 315° prior to interaction, to average values of 230°, 270°, and 345° upon phenothiazine binding. The corresponding values for Glu 87 vary from 175°, 75°, and 35° to 180°, 270°, and 340° (resp.). Conformational changes of a limited amplitude (less than 15°) also take place along the conformational angles τ of the phenothiazines upon complexation. The only exception relates to PMZ, which does not interact with any of the hydrophobic residues of the oligopeptide, and is found to retain its intrinsically preferred *gauche-gauche* conformation upon complexation. The results of the computations are reported in Table 1, which lists, for each bound phenothiazine derivative: the intermolecular phenothiazine-oligopeptide interaction energy ΔE,

TABLE 1

Values of the interaction energies in the optimized phenothiazine-oligopeptide complexes[a]

	PMZ	PZ	TFPZ	TFP
ΔE	−254.8	−263.7	−264.6	−264.4
E_{MTP}	−233.8	−243.8	−243.5	−236.6
E_{pol}	−21.5	−19.9	−19.8	−22.4
E_{CT}	−13.1	−17.5	−17.5	−16.1
E_{disp}	−23.5	−25.0	−27.0	−33.7
E_{rep}	37.1	42.5	43.2	44.4
δE_{cal}	31.1	31.4	31.4	28.7
δE_{phen}	0.0	6.8	5.6	5.3
E	−223.7	−225.5	−227.6	−230.4
δ	6.7	4.9	2.8	0.0
IC_{50} (μM)[b]	340	110	28	17

[a] See the text for definition. Energies were measured in kcal/mol.
[b] See Ref. 23 for determination of IC_{50} values.

together with its individual contributions; the conformational energy difference, δE_{cal}, between the intramolecular energy of the oligopeptide in the complex and that of its energy prior to complexation; the corresponding energy difference, δE_{phen}, for the phenothiazine; the resulting overall interaction energy, $E = \Delta E - \delta E_{cal} - \delta E_{phen}$; the difference, δ, of interaction energies with respect to the energy of the most stable complex taken as energy zero; and the experimental values of the inverse affinities of the investigated phenothiazine for CaM, expressed in terms of IC_{50} (μM), namely, the concentration of drug necessary to inhibit by 50% the activation of phosphodiesterase, as determined in Ref. 23.

Table 1 shows that, whereas neither ΔE, δE_{cal}, nor δE_{phen} alone can account for the proper order of binding affinities of the phenothiazines for CaM, it is their summation to obtain E that provides the correct experimental ordering, namely, TFP > TFPZ > PZ > PMZ.

The intermolecular interaction energies ΔE are much larger with PZ, TFPZ, and TFP than with PMZ. This is due to the absence of the additional stabilizing hydrophobic interactions in the PMZ complex and also to the presence of a methyl group on C'_2 of PMZ, which introduces some steric hindrances for the interaction of the cationic end with the glutamates, resulting in a slightly increased separation to these residues in the PMZ complex (see below). The introduction of δE_{phen}, however, markedly reduces the energy separation between the PMZ complex and the other phenothiazine complexes, since δE_{phen} is null in the former.

Let us compare in more detail the binding energetics of PZ, TFPZ, and TFP, the oligopeptide complexes of which have comparable configurations.

A small increase of ΔE, amounting to 0.9 kcal/mol, occurs upon passing from PZ to TFPZ, contributed by the incorporation of a $-CF_3$ group. This is due to the increase of the hydrophobic contribution to the binding, as evidenced by the increase of E_{disp}, with a concomitant but smaller increase of E_{rep}. The values of δE_{cal} are identical in the two PZ and TFPZ complexes. The value of δE_{phen}, by contrast, is smaller with TFPZ than with PZ by about 1.2 kcal/mol, indicative of a reduced *gauche-gauche* to *gauche-trans* energy separation upon incorporation of a $-CF_3$ substituent in the 2-position. As a result, the TFPZ complex is more stable than the PZ complex by 2 kcal/mol.

The ΔE values in the TFP and the TFPZ complexes are very

close, as are the corresponding values of δE_{phen}, and it is δE_{cal} that now accounts for the better complexation of TFP with respect to TFPZ. Owing to the more bulky size of the piperazinium end, the intermolecular separation between the protonated nitrogen and Glu 87 is slightly increased in the TFP complex with respect to the TFPZ complex, resulting in correspondingly smaller absolute values of E_{MTP} and of the short-range contribution E_{CT}. Conversely, hydrophobic interactions now take place between the piperazinium moiety and Val 91, which were not present with either TFPZ or PZ, the cationic end of which is trimethylammonium (see below).

These interactions are accompanied by a much larger dispersion energy contribution in the TFP complex, compensated to only a limited extent by the concomitant increase of the repulsion contribution. The polarization is also larger in the TFP complex, due to a larger value of the polarization of TFP than of TFPZ by the electrostatic field of the oligopeptide, whereas the polarization energy of the oligopeptide proper by the two phenothiazines has comparable values in the two complexes. As a result of the mutually compensating trends imposed upon by the separate first and second order energy contributions, the ΔE values in the TFPZ and the TFP complexes are comparable.

We surmise that an analysis of this kind can be of value when comparing, in more general situations, the binding specificities of a class of related molecules to a common receptor site model. However, given the rather elaborate expressions used for the individual energy contributions (30) and the evidence for the contrasting behaviors of the second order contributions E_{pol}, E_{ct}, and E_{disp} (which are lumped together in atom-atom potential functions or molecular mechanics procedures) in the different complexes, we think that it is necessary to resort to refined intermolecular procedures.

δE_{cal} has a larger value in the TFPZ complex than in the TFP complex. Owing to the bulkier size of the piperazinium end as compared to the trimethylammonium end, Glu 84 and Glu 87 come less close together in the TFP complex, resulting in a decreased value of their mutual electrostatic repulsions as compared to the TFPZ complex, a decrease which accounts for the predominant part of the difference of respective δE_{cal} values.

Let us now outline the characteristics of the optimized configurations derived for the PMZ, TFPZ, and TFP complexes (the stereochemistry of the PZ complex is similar to that of the TFPZ complex but for the absence of the $-CF_3$ group in the former).

PMZ complex (Fig. 3a). The binding of PMZ is principally stabilized by a hydrogen bond between the nitrogen-bound proton of PMZ and one oxygen, O_1, of Glu 87 (d_{O_1-H} = 1.80 Å). An additional hydrogen bond is found between the hydrogen linked to C'_2, on which the positive charge is partially delocalized, and one oxygen, O_1 of Glu 84 (d_{O_1-H} = 2.08 Å). In addition, two elongated hydrogen bonds are found between two hydrogen atoms of the C'_2 bound methyl group, H_a and H_b, and O_1 and O_2 of Glu 84 (d_{O_1-H} = 2.5 Å). The PMZ complex is not stabilized by any hydrophobic interactions with the oligopeptide side chains, in contrast to the complexes of the other, longer chain phenothiazines.

TFPZ complex (Fib. 3b). A very short hydrogen bond occurs between the nitrogen-bound proton and one anionic oxygen, O_1, of Glu 87 (d_{O1-H} = 1.60 Å). In addition, one hydrogen linked to C'_3, on which the positive charge is partially delocalized, bridges the two anionic oxygens of Glu 84, O_1 and O_2 (d_{O_1-H} = 2.02 Å; d_{O_2-H} = 2.13 Å). Hydrophobic interactions occur between Ala 88 and the C'_3—C'_2 methylene chain, characterized by distances between C_β of Ala 88 and C'_3 and C'_2 of 3.6 and 4.3 Å, respectively.

TFP complex (Fig. 3c). The overall configuration of the TFP complex is similar to the one found with TFPZ. A distinctive feature, however, consists of the establishment of additional hydrophobic interactions between the piperazinium ring of TFP and Val 91, a possibility which was not considered in Ref. 26.

There is a short hydrogen bond between the nitrogen-bound proton and O_1 of Glu 87 (d_{O_1-H} = 1.65 Å). One H linked to C'_3, on which the positive charge is partially delocalized, bridges the two anionic oxygen of Glu 84, the respective H—O distances being 1.84 and 2.23 Å. Similar to the TFPZ complex, hydrophobic interactions can be characterized between Ala 88 and the C'_3—C'_2 methylene chain.

This result can be correlated with the experimental result of Ref. 17, showing that in the sequence (90–102) of TnC, the ^1H NMR resonances of Ala 98 (which is homologous to Ala 88 in CaM) are significantly affected by TFP binding. Similar to the TFPZ complex, limited penetration of the CF_3 and the phenothiazine ring occurs between Phe 89 and Phe 92, with a closer vicinity of the $-CF_3$ substituent to Phe 92 than to Phe 89, whereas one F atom, F_c, resides in a crevice between Phe 89 and Ile 85. The interatomic distances between Ile 85 and TFP are large, comparable to the ones found in the TFPZ complex.

Experimentally, the binding of TFP to fragment 90–102 of TnC results in significant changes affecting the ^1H NMR resonance at 7.3 ppm, common to Phe 99 and Phe 102, which are homologous to Phe 89 and Phe 92 in CaM. The present model seems consistent with this result and implies, moreover, that TFP binding should be affecting more Phe 102 than Phe 99 in this TnC fragment.

The hydrophobic interactions between the piperazinium ring and Val 91 involve more particularly the ring nitrogen methyl group and the C_3'' H_2 methylene of the piperazinium, on the one hand, and both $C_{\gamma 1}$ H_3 and $C_{\gamma 2}$ H_3 methyl groups of Val 91 on the other. They are characterized by five intermolecular hydrogen-to-hydrogen distances in the range 2.2–3.0 Å.

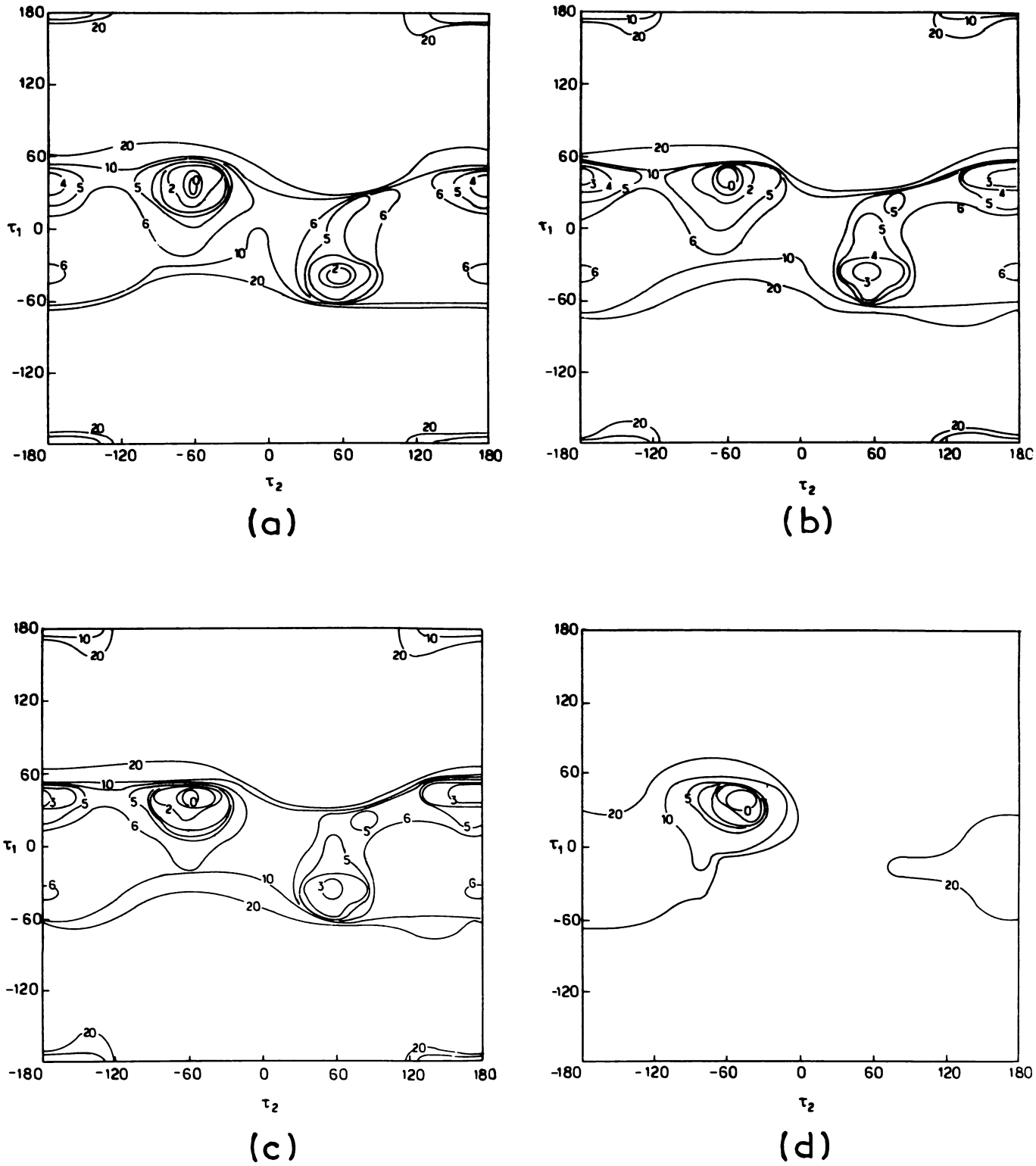
Experimentally, the binding of TFP to fragment 90–102 of TnC is accompanied by broadening and shifting of the leucine/isoleucine ^1H NMR resonances, which are centered at 0.9 ppm. In this fragment, Leu 95 and Ile 101 occupy the homologous positions of Ile 85 and Val 91 in CaM, respectively.

In the light of the present model, it is tempting to ascribe this result to a hydrophobic interaction involving the piperazinium ring and Ile 101, similar to the one occurring with Val 91 in CaM, rather than to a hydrophobic interaction of the phenothiazine ring with Leu 95 in TnC.

The results of the present study indicate that, in fragment 82–93 of CaM, the crucial residues involved in specific phenothiazine binding are two anionic residues, Glu 84 and Glu 87, two aliphatic residues, Ala 88 and Val 91, and the two aromatic residues, Phe 89 and Phe 92. Fragment 82–93 is the N-terminal part of CaM site 3. It bears a profound resemblance to the α-helical fragment 9–20 in the N-terminal part of site 1. The residues homologous to the six abovementioned ones are, respectively, Glu 11, Glu 14, Ala 15, Leu 18, Phe 16, and Phe 19. They could adopt the same respective conformations as the former sequence, upon phenothiazine binding, the sole change

Appendix

Conformational Energy Maps for: (a) PMZ, (b), PZ, (c) TFPZ, and (d) TFP



being the replacement of Val 91 by the related Leu 18. In contrast, it is significant to observe that Glu 82 and Glu 83, which are not directly involved in the binding, are now replaced by two hydrophobic residues, Ile 9 and Ala 10. The homology of these two N-terminal parts of CaM sites 1 and 3 was stressed in (17, 26) as an indication for the additional binding site of CaM being the helical region of the N-terminal part of CaM site 1. The stereochemical model derived from the present study is in line with this proposal. There is no homology between the sequence 82–93 and those of the C-terminal part of CaM sites

2 and 4, which encompass, respectively, residues 61–76 and residues 134–148. Binding of phenothiazine derivatives to these two sequences, if it actually occurred in CaM *and* displayed specificity (for discussion, see, e.g., Ref. 17 and references therein), would obviously imply a stereochemistry completely different from that investigated here.

Inherent to this study were the assumptions of the independence of the two phenothiazine-binding sites, which are located in two distinct parts of the protein, the occupancy of the four Ca^{2+}-binding loops by Ca^{2+}, and that, on account of structural constraints, no major conformational rearrangements will occur with respect to the preexisting structure of CaM as revealed by X-ray diffraction (20). In the latter, the proximity to fragment 82–93 of aromatic residues of the C-terminal part, namely, Tyr 138 and Phe 141, could possibly enhance further the binding by means of additional hydrophobic contributions (see, e.g., Ref. 38). The stereochemical model derived here indicates that part of the phenothiazine ring remains accessible for such additional interactions. However, their presence may not be an absolute requirement for phenothiazine binding since fragment 77–124 of CaM, which lacks them, is fully able to complex phenothiazines (39).

Conclusions

The present theoretical computations have enabled us to account, in a *quantitative fashion*, for the experimental ordering of CaM binding by a series of four related phenothiazine derivatives, PMZ, PZ, TFPZ, and TFP. As a starting point for our study, we resorted to an explicit proposal (26) limiting one of the phenothiazine binding sites to sequence 82–93, based on a structural study of trifluoperazine binding to the related protein TnC (17). A detailed stereochemistry for the different phenothiazine complexes could be derived. Thus, in the TFP complex, a distinctly tighter binding to Glu 87 than to Glu 84 could be evidenced, together with hydrophobic interactions involving Ala 88, Val 91, Phe 92, and, to a lesser extent, Phe 89.

Let us finally point out that, in this study we were principally interested in the *comparative* binding affinities of the investigated phenothiazines to CaM. As underlined in preceding studies (see, e.g., Ref. 40), the absolute values of interaction energies of the phenothiazine derivatives should not be correlated with experimental values of their binding enthalpies to the CaM oligopeptide (unavailable at present). This would require one to account, in rather extension fashion, for solvation/desolvation effects (see, e.g., Ref. 41).

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