A STUDY OF CALMODULIN AND ITS INTERACTION WITH TRIFLUOPERAZINE BY HIGH RESOLUTION ^{1}H NMR SPECTROSCOPY

R. E. KLEVIT, B. A. LEVINE and R. J. P. WILLIAMS
Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, England

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1. Introduction

The antipsychotic drug trifluoperazine (TFP) is known to be an effective inhibitor of the calcium-binding activator protein, calmodulin (CaM). It has been shown that 2 mol TFP bind to 1 mol CaM with $K_d \sim 10^{-6}$ M [1]. In [2] ^{113}Cd NMR was used to study the effects of TFP binding to CaM on the cation binding sites of the protein. It was found that the binding of 2 mol drug to 1 mol protein affected all 4 cation-binding sites.

We have been using high resolution ^{1}H NMR to study the conformation of calmodulin in solution under varying conditions. Our observations on the protein itself agree with those in [3]. Here we report the effects of drug binding on the ^{1}H NMR spectrum of calmodulin.

2. Materials and methods

Calmodulin was prepared from bovine brain essentially by the method in [4]. The purity of the protein was checked by polyacrylamide gel electrophoresis, in the presence and absence of SDS, and by the ability of the protein to activate activator-deficient cyclic nucleotide phosphodiesterase.

Pulsed Fourier transform ^{1}H NMR spectra were monitored using a modified Bruker console, an Oxford Instruments superconducting magnet and a Nicolet 1085 computer at 270 MHz or a Bruker WH300 spectrometer at 300 MHz. Quadrature detection was used. All spectra were obtained at 37°C. The protein solutions were made up in 5 mM Tris, 0.2 M KCl, pH 7.4 in D_2O. Although we have made a full study of the protein on its own we shall not report our assignments of the calmodulin spectrum itself since they agree with those in [3]. Methionine resonances which are important in the description of the results were recognised as singlet methyl groups in the region of 2.0 ppm.

3. Results

3.1. *The addition of trifluoperazine to calmodulin*

Aliquots of a 30 mM solution of TFP in D_2O were successively added to a 2 mM solution of calmodulin which was fully bound with calcium ($[Ca^{2+}]$ = 10 mM; [KCl] = 0.2 M; pH 7.4). Some of our observations are shown in fig.1. The continuous shifts of peaks with little broadening shows that the system is in fast exchange on the NMR time scale. There was little effect on the aromatic region of the protein spectrum. The most downfield phenylalanine resonance (7.35 ppm) moved upfield, disappearing under other phenylalanine resonances at 7.26 ppm on the addition of 1 mol equiv. TFP. This resonance is associated with the weak sites of calcium binding (in preparation). A new sharp resonance grew in intensity and first appeared clearly in the TFP:CaM = 1:1 spectrum at 6.97 ppm. The intensity of this peak increased further and continuously throughout the titration and shifted upfield to 6.70 ppm in the TFP:CaM = 2:1 spectrum. There was also a continuous increase of intensity at around 7.00 ppm and this peak shifted continuously upfield to 6.89 ppm during the titration. These two resonances are due to the aromatic protons of the drug molecule which are centred around 7.20 and 7.03 ppm in the protein-free drug spectrum. These peaks may hide small changes in the protein spectrum.

The changes to the aliphatic region of the calmodulin NMR spectrum caused by the drug binding were

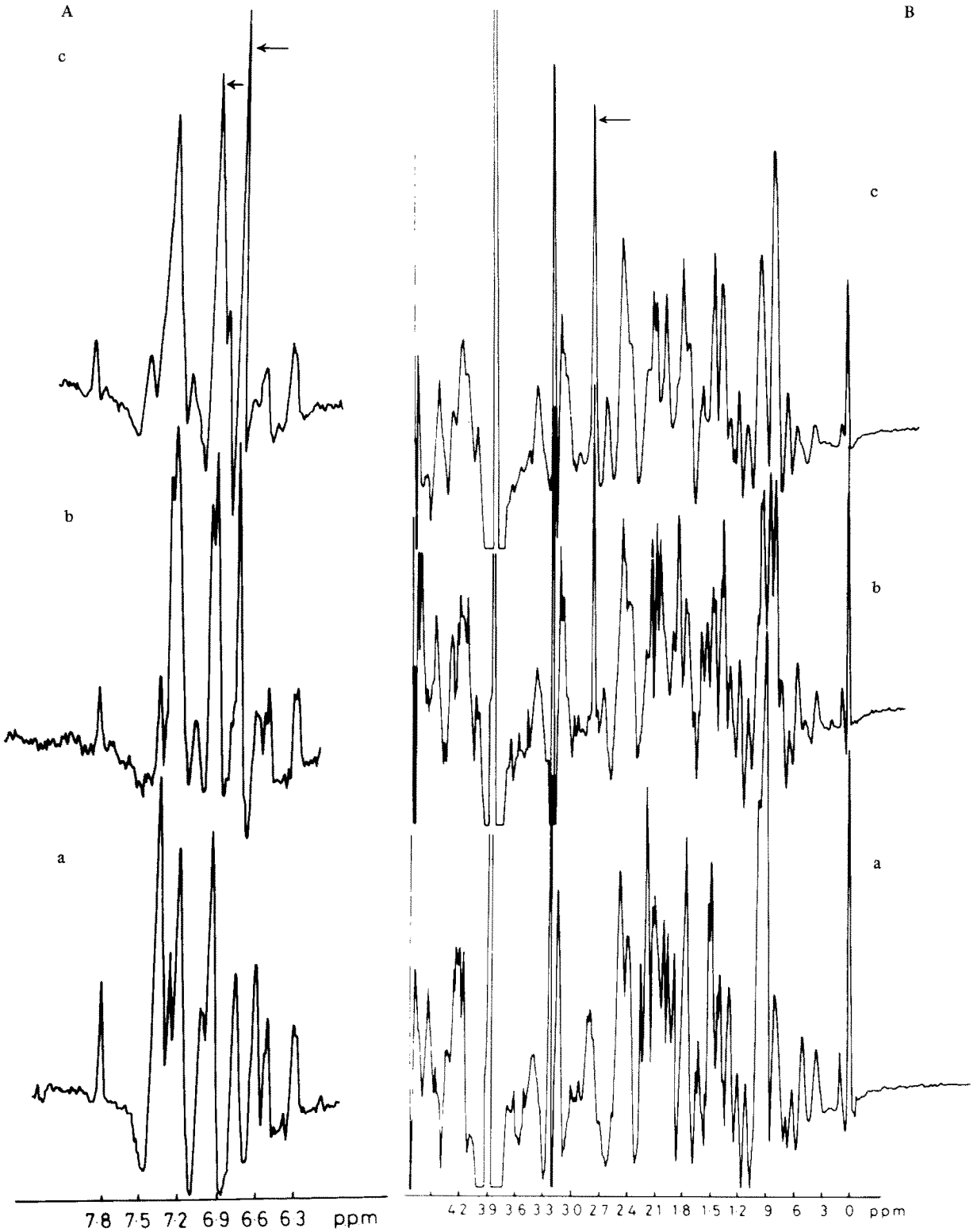

Fig.1. (A) The aromatic region; (B) the aliphatic region of the convolution difference spectra at 270 MHz: (a) calmodulin; (b) TFP:CaM = 1:1; (c) TFP:CaM = 2.1. Arrows indicate the resonances due to protons of TFP. Chemical shifts are relative to TSS = 0.0 ppm.

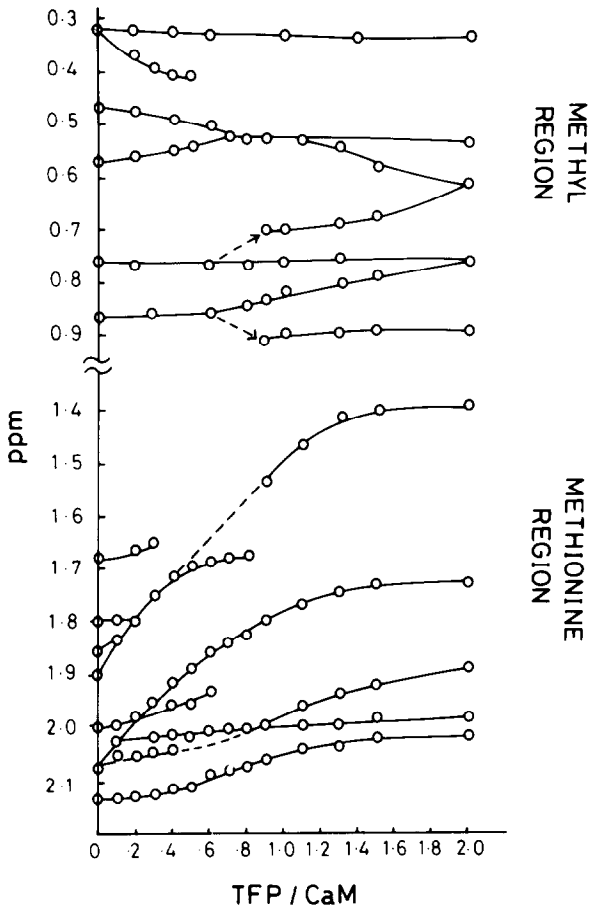

Fig.2. The chemical shifts of some of the aliphatic resonances in the ^1H NMR spectrum of calmodulin during titration with TFP binding.

more dramatic. The affected resonances may be categorized into 3 types of behaviour (fig.2):

(i) Those which changed during the addition of 0–1 mol TFP/mol CaM;
(ii) Those which changed during the addition of 1–2 mol TFP/mol CaM;
(iii) Those which changed continuously throughout the entire titration.

In the first half of the drug titration, the following observations were made. The most upfield shifted absorption peak (0.31 ppm) split gradually into two peaks. Part of the intensity remained at 0.31 ppm and part shifted downfield towards the major methyl peak. Three singlet resonances due to the methyl group of methionine residues, which are resolved in the drug-free spectrum at 1.90, 1.85, and 1.80 ppm, merged into a single resonance and at least two of the resonances titrated upfield to around 1.68 ppm. At the same time, intensity was lost from the region of 1.70 ppm in the drug-free spectrum. This resonance has been assigned to the β-CH_2 protons of lysine residues (unpublished). In the TFP:CaM = 1:1 spectrum, both the lysine and the methionine resonances at 1.68 ppm were somewhat broad. Another singlet resonance from a methionine methyl group at 2.00 ppm broadened and disappeared from the spectrum. Some smaller changes occurred elsewhere in the spectrum.

During the second half of the drug titration further observations were made. A peak appeared upfield of the major methyl peak and continued to shift upfield to 0.60 ppm. At the same time, part of the major methyl peak (0.86 ppm) shifted upfield to 0.76 ppm. As that intensity shifted upfield, the residual intensity was left behind at 0.90 ppm, so that in the TFP:CaM = 2:1 spectrum there are two large methyl peaks of approximately equal intensity. At TFP:CaM $>$ 1, the peak at 3.04 ppm due to the ϵ-CH_2 protons of lysine residues (unpublished) broadened.

There were several other resonances which changed continuously throughout the whole titration. The most dramatic of these was an easily seen methionine singlet resonance which titrated upfield in fast exchange from 2.07–1.71 ppm, while a second resonance, possibly another methionine, moved from 1.85–1.40 ppm. Two other methionine resonances also titrated upfield with smaller shifts of 0.19 and 0.12 ppm, most of the shifts taking place in the second half of the titration. The upfield-shifted methyl resonances at 0.57 ppm shifted further upfield, joining with the resonance from 0.47 ppm to form a sharp peak at 0.54 ppm in the 1:1 spectrum from which, at higher TFP:CaM ratios, some intensity shifted downfield to 0.60 ppm. A sharp peak appeared at 2.70 ppm and grew continuously. This resonance appeared as a singlet in the Carr-Purcell spectrum of the drug–protein complex and is due to the terminal N-CH_3 group of the drug molecule. In the absence of protein this peak appears at 2.76 ppm.

3.2. *Nuclear Overhauser effect difference spectroscopy*

Nuclear Overhauser effect (NOE) difference spectroscopy was performed on the final TFP–CaM complex. When the methyl resonance at 0.60 ppm was irradiated, 3 aromatic resonances showed a negative NOE. These were the 2 drug resonances at 6.70 and 6.89 ppm and 1 protein resonance at 7.05 ppm. When the methyl resonance at 0.76 ppm was irradiated,

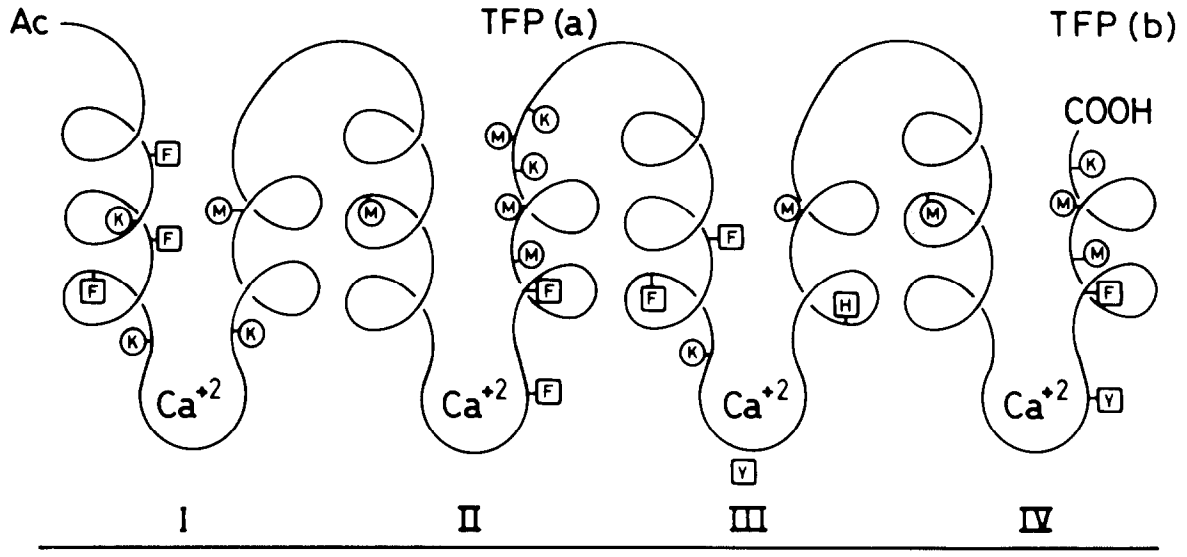

Fig.3. A diagramatic representation of the sequence of calmodulin. Each domain (I–IV) includes an EF hand. All aromatic, methionine, and lysine residues are marked. The proposed TFP binding sites are noted.

the 2 drug peaks and a phenylalanine resonance at 7.19 ppm showed negative NOE. The protein peaks observed in these NOE experiments correspond to regions in which the protein spectrum changed on drug binding.

4. Discussion

The NMR titration of calmodulin (CaM) with trifluoperazine (TFP) has 2 steps which have quite distinct effects on the spectra of the protein. This result agrees with [1], showing that the binding ratio is 2:1. The on/off rate of both drug molecules are fast on the NMR time scale and we can state that despite strong binding the off rate constant is $\sim 10^2\ s^{-1}$ which compares well with the rate in [2] of $\sim 10^3\ s^{-1}$.

This NMR study permits us to make certain structural conclusions. We do this in the light of a diagramatic representation for calmodulin which has 4 EF hands (fig.3) although we are not able to say in what order the hands are put together in the folded form. The diagram shows the location in the sequence of several important residues.

The first important observation is that there is a relatively small effect in most regions of the NMR spectrum despite the fact that TFP is an aromatic molecule. Certain resonances are totally unaffected; including both tyrosines, the trimethyllysine, and much of the phenylalanine spectrum. One phenylalanine is slightly affected however. We concluded that there is only a small interaction between the drug and any of the aromatic side-chains. The fact that the resonances of the drug itself are largely unaltered again indicates that it does not come close to the aromatic side-chains of the protein; see below.

The methyl region of the NMR spectrum of the protein is grossly affected and very differently by the first and second drug molecules. The biggest shifts for the binding of the first molecule are on the methionine (especially marked), one or two ring-current shifted methyl resonances, and on lysines. Reference to the sequence (fig.3) shows that a possible explanation lies in binding in the regions of the top of domains II or IV. The second drug molecule has a smaller effect everywhere in the spectrum except in the unshifted methyl group region. Clearly, the aromatic side-chains of the protein are not involved, but once again methionine resonances are greatly altered. We indicate the possible parts of the sequence for the binding of the second molecule of TFP in fig.3. We note that the sequences of domains 2 and 4 are homologous.

The NOE experiments confirm the studies using the shifts of protein resonances. The most striking observation is the strong NOE from the upfield shifted methyls to the drug aromatic protons.

Reference to fig.3 shows that the drug-binding sites are not close to the calcium-binding sites which

are known to carry aromatic residues. The fact that Forsén et al. (2) observed effects from drug-binding upon ^{113}Cd (in place of calcium) resonances implies that the remote binding of the drug relays effects to these sites. However the drug does not alter much the binding constant for calcium. It would appear that the drug binds to a region of the calmodulin involved in association with receptor proteins since the methionine-rich region of calmodulin is near the homologous transmission region of troponin C uncovered in [5]. Elsewhere we shall report a very similar reaction of troponin C with TFP.

Acknowledgements

In order to study the NMR of calmodulin in the detail with which we have been concerned, we have required large amounts of purified protein. We wish to thank Dr R. H. Kretsinger, Professor S. V. Perry, and Dr T. C. Vanaman for their generosity in supplying us with protein. We also wish to thank Dr M. P. Esnouf for his help and advice with our own preparations of calmodulin. A detailed description of the preparation of the protein and characterisation will be published elsewhere.

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