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Enhanced Ligand Affinity for Receptors in which Components of the Binding Site are Independently Mobile

Running title: Receptors with Mobile Binding Sites

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Summary

Using calmodulin antagonism as a model, it is demonstrated that under circumstances where binding sites are motionally independent, it is possible to create bifunctional ligands that bind with significant affinity enhancement over their monofunctional counterparts. Suitable head groups were identified using a semi-quantitative screen of monofunctional tryptophan analogues. Two bifunctional ligands were synthesised, which contained two copies of the highest affinity head group tethered by rigid linkers. The bifunctional ligands bound to calmodulin with a stoichiometry of 1:1 and with an affinity enhancement over their monofunctional counterparts, the latter binding with a stoichiometry of 2:1 ligand to protein. A lower limit to the effective concentrations of the domains of calmodulin relative to each other (0.2-2 mM) was determined. A comparable effective concentration was achieved for bifunctional ligands based on higher affinity, naphthalene sulphonamide derivatives.

Introduction

A successful strategy to design ligands to biological macromolecules, which have both high affinity and high specificity, is to link together two lower affinity ligands [1-2]. Such an affinity enhancement, however, is by no means assured [3-4]. It depends on (i) how much the binding conformation of the macromolecule is perturbed away from the optimum given the restraints imposed by accommodating the linked compound, (ii) how large an entropic loss in the target molecule is required by the binding, and (iii) how much flexibility the linked compound is required to lose in the linker on binding. All of these components combine to determine the effective concentration for the second interaction. The magnitude of this effective concentration, C_{eff} , is reflected in any enhanced affinity of the bifunctional ligand over the corresponding monofunctional ligands, and is given by:

$$C_{\text{eff}} = \frac{K_d^A K_d^B}{K_d^{AB}} \quad \text{Equ. 1}$$

where A and B represent two moieties of a bifunctional ligand AB, for which the dissociation constants as monofunctional ligands (K_d^A and K_d^B) are known. To achieve simultaneous binding of, for example, two domains to one ligand, the value of C_{eff} needs to be larger than the dissociation constant of the weaker binding monofunctional moiety. In this scenario, C_{eff} is also the concentration at which the formation of linear polymers of receptors linked by bifunctional ligands begins to compete effectively with 1:1 binding. Thus if the value C_{eff} can be determined (or at least a lower limit of its value), this provides a quantitative measure of the minimum affinity that must be achieved by the component moieties of a bifunctional ligand, if binding in a bifunctional mode is to provide an improvement in affinity. As an example, for a protein that binds to a monofunctional ligand with a dissociation

constant in the micromolar range, a C_{eff} value greater than micromolar will ensure that the simultaneous participation of both domains in a complex with the target is the preferred state (Equation 2).

$$\text{Enhancement of bifunctional binding} = \frac{K_d^A}{K_d^{AB}} = \frac{C_{\text{eff}}}{K_d^A} \quad \text{Equ. 2}$$

To date, applications of linked bifunctional ligands for biological macromolecular receptors have focussed on macromolecules containing adjacent ligand binding sites that are in a fixed positional relationship to one another. Reported examples of linked ligands include a wide variety of multi-substrate analogues, including bi-substrate analogue inhibitors for a range of enzymes. For example, the design of linked ligands was developed empirically based on models of transition state complexes involving two substrates and has led to affinity enhancements of 2 - 5 orders of magnitude [5-10]. Multi-substrate ligands also largely involve rigid linkers, including examples that have been either designed [11] or selected by combinatorial methods [12] to be closely complementary to protein sites that, again, have fixed positional relationships. An alternative strategy, developed for a homo-multimeric protein with binding sites in fixed positional relationships [13], utilises highly flexible linkers between two ligand moieties. The linkers do not become immobilised on binding but determine, according to their length, the effective concentration of the two head groups.

The use of bifunctional ligands for multidomain proteins that have ligand binding sites that are not fixed in position relative to each other, though, has been largely overlooked [14]. However, a corollary of the successful use of flexible ligands for

rigid binding sites [13] is that it should be achievable to attain increased binding affinity to proteins with motionally independent binding sites using ligands connected by fixed linkers, if a sufficiently high effective concentration can be attained. Although effective concentrations as high as 10-100 M have been reported for complexes between bidentate ligands and non-protein receptors in which the binding site orientations are fixed, these values are greatly reduced for the equivalent compounds in complexes where the binding sites have relative motion [15]. In addition, the reported effective concentrations attained between linked ligands and proteins rarely approach the values attained for non-protein receptors, even when the binding sites have relatively fixed orientations and positions [1]. Therefore, if bifunctional ligands with enhanced affinity are to be developed for binding sites that are not in fixed relative positions, it is important to establish a strategy by which the effective concentration of a second interaction can be readily measured in order to ascertain whether linked ligands are likely to be an improvement over their monofunctional counterparts.

Here we show how a limit on C_{eff} can be readily established for a protein, in which the ligand binding sites are motionally independent, using small, rigid linked ligands. We illustrate the strategy using the ubiquitous calcium signal transducer calmodulin (CaM) as a representative example (Figure 1). CaM comprises of two highly homologous domains, each of which contains a hydrophobic surface, exposed on the binding of calcium ions [16-18], and which binds to a variety of hydrophobic, small molecule antagonists [19-20]. The binding of numerous such monofunctional ligands has been characterised both thermodynamically and structurally [21-25]. Motional independence between the two domains when CaM is unliganded has

been clearly demonstrated [26-28], and the protein is known to use both domains in the binding of recognition sequences of target proteins in a closed conformation [29-31], implying that a high value of C_{eff} can be achieved despite the relative motion of the unliganded form. However, the value of C_{eff} has not been established for the peptide (or antagonist) complexes of CaM and the attainment of an equivalently high effective concentration with small linked ligands is not guaranteed for the reasons outlined above. In terms of ligand design, the structural similarity of the two antagonist binding sites in CaM allowed us in this case to utilise symmetric bifunctional ligands, and the established ability of CaM to accommodate target peptides of different lengths [30] enabled us to synthesise a limited number of bifunctional ligands with predominantly rigid linkers of different lengths between the two recognition moieties. In general, though, the strategy can be equally applied to asymmetric ligands and a far wider series of linkers that are synthesised either individually, or combinatorially and selected on the basis of affinity for the target protein.

Results

In order to establish a value for C_{eff} using bifunctional ligands, the dissociation constants of the monofunctional components from which the bifunctional ligand is constructed need only to exceed C_{eff} . In other words, in the scenario where C_{eff} is high (e.g. in the milli-molar range), and therefore where the use of bifunctional ligands could be highly advantageous, the affinity for the target of the monofunctional components used to measure C_{eff} can be relatively low, i.e. the ligands that are linked can be relatively poor models of the optimum binding moiety or head group.

Peptide-mimic ligands – In the case of CaM, the first-generation of bifunctional ligand that was investigated was based simply on the amino acid tryptophan, which is a component of many natural CaM target peptides, but only part of the moiety that contacts the hydrophobic surface on each of the CaM domains. Tryptophan is a convenient choice from a chemical perspective in that it possesses spectroscopic properties that are not present in CaM and it has the potential for simple functional group modifications through which to attach a linker. Tryptophan also broadly conforms to the CaM antagonist paradigm [19], which constitutes a hydrophobic, aromatic head with a basic tail. For CaM, the number of compounds synthesised could be limited owing to the wealth of structural information already available, and therefore the measurement of C_{eff} is simplified experimentally by removing the need to make many compounds and separate them according to their affinity. The three-dimensional structures of CaM - target peptide complexes show that tryptophan sidechains bind within a well-defined pocket in the hydrophobic surface of each CaM domain, the two pockets being separated by more than 20 Å. Thus it was likely that bifunctional ligands based on two tryptophan moieties should be accommodated by CaM without the introduction of substantial strain into the protein.

Using 1D ^1H NMR and ligand fluorescence, an initial semi-quantitative screen of a family of homologous monofunctional tryptophan-based ligands, MT1 - MT6 (Figure 2A), was carried out to select which variant of the tryptophan moiety was the most suitable head group for the construction of bifunctional ligands (Figure 2B). The most reliable estimate of the relative binding affinities of the ligands was obtained using pulsed gradient spin echo (PGSE) NMR experiments [32]. These were

performed on samples with ligand:protein concentration ratios of 2:1, and the spectra were edited according to the translational diffusion properties of the protein. The resulting resonances reflect the population of ligand that is not bound to the protein. In Figure 3A three examples of such diffusion-edited spectra are shown, in comparison with the spectra of the free ligands. The chemical shift changes that occurred on addition of protein to ligand solutions were in the NMR fast exchange regime. Resonances from both CaM domains were affected simultaneously by ligand binding and followed identical binding isotherms indicating similar binding affinities for the two domains, as observed previously for a range of monofunctional ligands [23]. In Figure 3B are summarised the amplitudes of the chemical shift changes observed on binding for representative nuclei in both the protein and the ligand. These data illustrate the general trend that larger changes in chemical shift are associated with higher affinity binding, as determined by the PGSE data. For ligands MT1 and MT6, the very small perturbation of chemical shifts and the high residual signal intensity in diffusion-edited spectra indicates the absence of significant interaction with CaM at milli-molar concentrations. For MT2 - MT5 all probes indicate significant interaction with CaM, with MT2 and MT3 showing the strongest effects. The MT2/MT3 type head group was therefore selected for the synthesis of bifunctional ligands.

Based on the structures of CaM in complex with peptides models of protein targets [29-31] the approximate lengths of linkers that would accommodate the domain separation of a closed form of CaM were estimated (Figure 1). Two bifunctional ligands (Figure 2B) were synthesised, each containing two identical tryptophan head groups tethered by predominantly rigid linker groups. A quantitative analysis of the

binding affinity was initially carried out using NMR for the ligands BT1, MT2, MT3, and MT7, the last being a 'mono+linker' ligand designed to probe the effects attributable solely to the linker group (Figure 2). Again, the binding of all ligands to CaM was in the NMR fast exchange regime. Examples of changes in chemical shift as the ligand:CaM ratio is increased are shown for a mono and a bifunctional ligand in Figure 4A, and the derived dissociation constants and stoichiometries for each are summarised in Table 1.

Importantly, ligand BT1 has a 1:1 stoichiometry with CaM, compared to the 2:1 stoichiometry of the monofunctional ligands, including MT7. For BT1, protein chemical shift changes were complete by 1.0 ligand equivalents indicating that the dissociation constant is at least two orders of magnitude less than the protein concentration (i.e. the strong binding limit). None of the monofunctional ligands were at this limit. Although an accurate dissociation constant for BT1 cannot therefore be determined directly from this titration, the weakest affinity possible corresponds to a dissociation constant of $\sim 10 \mu\text{M}$, whereas the fast exchange of NMR resonances from the free and bound species indicates that the strongest affinity possible is no greater than $1 \mu\text{M}$. NMR monitored titrations at CaM concentrations below the strong binding limit were supportive of a dissociation constant in the range $1 - 10 \mu\text{M}$, but a more precise value could not be obtained owing to limitations in sensitivity. Stoichiometric addition of bifunctional ligands to CaM induces its transformation to a closed form (Figure 1), as determined by small-angle X-ray scattering. For example, the radius of gyration of CaM falls from 21.7 \AA to 18.7 \AA on binding of BT1 (Figure 5). This change compares closely with the fall in the radius of gyration when comparing previously reported values for unliganded

CaM and for 1:1 MLCK-I+CaM [33-34].

Although the incorporation of tryptophan moieties was intended to allow the use of ligand fluorescence for the determination of higher affinity dissociation constants, the indole fluorescence is completely quenched by the linker group in BT1, and largely quenched in BT2. Only a low intensity fluorescence signal with maxima at 380 and 460 nm could be observed for the latter. From a titration of CaM into BT2 monitored by fluorescence (Figure 4B), the measured dissociation constant for the 1:1 interaction is approximately 9 μ M (Table 1).

For the bifunctional ligand BT1, ligands MT3 and MT7 can be used to represent its two component moieties in the determination of C_{eff} . BT1 binds with an affinity enhancement of between 1 and 2 orders of magnitude over MT3, and a factor of 5 over MT7 (Table 1). Importantly, the stoichiometry difference between the bifunctional and the mono+linker ligand, MT7, indicates that it is the second head-group moiety, rather than the presence of the linker group, which causes the bifunctional binding. Using the derived dissociation constants (Table 1), C_{eff} for the domains of CaM for BT1 is in the range \sim 0.2 - 2 mM. The length of the linker groups in BT1 and BT2 was found to make little difference to the binding affinity, as predicted from the ability of CaM to accommodate peptides with different relative positions of the sidechains that bind to its hydrophobic surfaces.

In principle, the major contribution of C_{eff} to the binding affinity is usually ascribed to an entropy term that favours binding for bifunctional ligand:CaM complexes. To test

whether the increase in binding affinity for the bifunctional ligands is largely the result of such an entropy term, isothermal titration calorimetry (ITC) was used to measure the changes in enthalpy (ΔH), and hence entropy (ΔS), on binding for ligands MT2 and BT1. The dissociation constants measured by ITC (Table 2) match within error those measured by other methods (Table 1). Over the temperature range 8-37 °C, ΔH and ΔS were negative for binding of both ligands, and the negative values of ΔC_p imply a significant hydrophobic contribution to the binding free energy. The value of ΔH for the bifunctional ligand is twice that for the monofunctional ligand (within error), which suggests that there is no substantial change in the individual binding interactions at the two sites. However, ΔS for binding the bifunctional ligand is also within error twice that for the monofunctional ligand, so it is not possible to identify an unambiguous entropic contribution to C_{eff} .

Dissecting these factors is a mayor challenge, so it is impossible to reliably assign the cooperativity to an entropy or enthalpy effect in this system.

The problem is that there are several different factors that can contribute to the observed enthalpy and entropy changes. For the monofunctional ligand these are:

1. Binding interactions. These could make a favourable contribution to ΔH .
2. Hydrophobic effects. These could make a favourable contribution to ΔS and an unfavourable contribution associated with the desolvation of polar groups.
3. Restricting intermolecular motion. This will make an unfavourable contribution to ΔS [35].
4. Conformational changes in the protein and /or the ligand. These can make

favourable or unfavourable contributions to ΔH and ΔS . Structural tightening could make ΔH and ΔS more negative or structural loosening would make ΔH and ΔS more positive. These compensatory changes in ΔH and ΔS are often very large relative to the size of the effect discussed here [36].

For the bifunctional ligand, the magnitude of these effects will differ from the monofunctional system. The contributions from 1 and 2 should be approximately double those for the monofunctional ligand and 3 should be similar. However, in the bifunctional case, there is additional restriction of the conformations of the linkers between the two protein domains and the tryptophan moieties which will compensate the entropic advantage of the bifunctional ligand to some extent.

Naphthalene ligands – Despite the relatively low affinity of tryptophan - based monofunctional ligands, the range measured for C_{eff} is sufficient to lead to a modest affinity enhancement for the equivalent bifunctional ligands. However, if this range of C_{eff} can be maintained, it follows from equation 1 that a larger enhancement of affinity should be achievable for bifunctional ligands starting from monofunctional moieties with higher affinities than MT1 - MT7. To demonstrate this, a second generation of bifunctional ligands, BN1 and BN2 (Figure 2C), was synthesised in which the linkers were based on the tryptophan-based ligands, but two naphthalene sulphonamide head groups were used in place of the tryptophan moieties. Naphthalene sulphonamides bind to the same sites of CaM as tryptophan

derivatives, as indicated by the distribution of chemical shift changes in the protein on addition of ligand [23-24], and are a feature of numerous monofunctional CaM antagonists that bind with dissociation constants in the range 1 - 10 μ M [19, 23-24], i.e. 1-2 orders of magnitude more strongly than the monofunctional tryptophan derivatives. The linker groups were chosen to give overall ligand lengths comparable to the tryptophan-based bifunctional ligands. The dissociation constants for BN1 and BN2 were determined to be 70 nM and 10 nM, respectively (Table 1), using changes in intrinsic fluorescence of the ligand at 550 nm on titration of CaM into a solution of the ligand. The stoichiometry for each binding event was confirmed as 1:1 using a titration of ligand to CaM at concentrations of ten times the determined dissociation constants.

Thus, the simple bifunctional derivative BN2 binds to CaM with a 1:1 stoichiometry and an affinity approaching that of peptides corresponding to the natural recognition target of CaM. This affinity is some 2 - 3 orders of magnitude stronger than that of any monofunctional ligand equivalents and indicates that this second generation of ligand was no more limited in C_{eff} by the linker design than the tryptophan-based bifunctional ligands. In other words, on the basis of the range determined for C_{eff} using the tryptophan-based bifunctional ligands, it was straightforward to design a bifunctional ligand that greatly exceeded its monofunctional counterparts in affinity and that binds CaM in competition with its physiological partners.

DISCUSSION

From the example of ligand binding to CaM, it is clear that ligands to biological macromolecules with enhanced affinity can be derived by the linking together of two lower affinity ligands even when the binding sites are mobile relative to each other. The degree of affinity enhancement is related to the effective concentration, C_{eff} , of the second binding event, a quantity that can be readily determined using simple, low affinity head groups connected by relatively rigid linkers. The monofunctional tryptophan-based ligands that were linked in the present study and that were sufficient to determine the value of C_{eff} for CaM have a considerably lower affinity for CaM (Table 1) even than traditional monofunctional CaM antagonists, such as J-8 and trifluoperazine. Relative to the latter, binding is likely to be disfavoured by the indole NH group of the tryptophan moiety which constitutes a hydrogen bonding potential that is not present in the other ligands, and that is not satisfied by the ligand-binding site. The absence of a significant change in the chemical shift of the indole NH resonance on binding (see Figure 3B) supports this, indicating that its solvation state is retained in the complex.

Paradoxically, ligand MT6 is melatonin, a neurotransmitter reported to inhibit CaM dependent activation of some enzymes [37] and to have a pico-molar dissociation constant for CaM [38], i.e. even stronger binding than the nano-molar affinities of target enzymes for CaM [39]. However, in agreement with a previous study [40], the results obtained here reveal a dissociation constant weaker than milli-molar for melatonin, and indicate that substitution of the indole ring further disfavours binding

to CaM. Furthermore, the weak interaction of ligand MT1 with CaM compared to the stronger binding of ligands MT2 and MT3 suggests that it is not the presence of a positive charge that favours CaM binding, as proposed in the paradigm molecule [19], but that a negative charge is detrimental to binding.

The range of C_{eff} values determined here (0.2 - 2 mM) represent a lower limit for that achievable for the domains of CaM. Despite each linker in the bifunctional ligands being predominantly rigid, six rotatable bonds separate the two indole moieties in the tryptophan derived bifunctional ligands, and four in the naphthalene sulphonamide bifunctional ligands. Although this flexibility may help alleviate constraints imposed by suboptimal orientation and separation of the head groups, any restriction of rotors would ameliorate the benefits, leading to a compromise in affinity enhancement and a corresponding lowering of C_{eff} . Naturally, ligands requiring the restriction of too many rotors or with inappropriately oriented or separated head groups will have effective concentrations below the dissociation constant of the corresponding monofunctional ligands, whereby 2:1 stoichiometry binding will dominate the equilibrium, and any affinity enhancement is lost. Conversely, more rigid ligands with appropriate head group orientation and separation have the potential for considerably enhanced affinity over that reported here.

As would be expected for many multidomain proteins, a relatively high effective concentration of the two domains of CaM is reflected in its mode of action. The coupling of the two domains by the tether limits the free energy loss resulting from the conformational selection required to bring the two domains together on formation

of complexes with the target proteins. The cooperative behaviour of the two domains at cellular calcium levels is also further moderated by the differences in their calcium affinity [41]. Moreover, CaM accommodates multiple target proteins that present a variety of binding regions. Interdomain flexibility is a convenient means of relaxing the constraints on substrate specificity while tethering maintains a large enough effective concentration to illicit co-operative binding. The CaM system also restricts its free energy loss on co-operative binding of the two domains by the behaviour of the tether in the CaM-peptide complexes. On binding peptides, not all of the region between the two domains is conformationally frozen, evidenced by the absence of electron density in this region in the crystal structure of CaM - CaMKII peptide complex [30]. Over-restriction of the tether would diminish the affinity of the two-domain complex in an analogous manner to the over-restriction of rotamers in the bifunctional ligands described above.

Significance

The results presented here for CaM clearly illustrate the value in utilising linked ligands for enhancing the binding affinity of ligands to proteins with motionally independent binding sites, and the value of quantifying a lower limit on the achievable effective concentration for multi-dentate interactions using simple headgroups with, in this case, millimolar dissociation constants. While the mechanism of action of CaM strongly hints at the potential advantage of using bifunctional ligands, and which turned out to be highly successful, many systems for which an equivalent strategy could be equally successful will not be so well-defined in terms of structure and affinity measurements. Nevertheless, knowledge about any independent ligand binding properties, regardless of any apparent relative

motion of the parts of the protein involved in that binding, should be sufficient to design simple linked ligands with which to test the achievable values of C_{eff} in an analogous manner to that described above for CaM. In other words, the observation of relative motion between potential ligand binding sites should not deter the investigation of linked ligands as high affinity derivatives. The nature of the tether between the ligand binding sites can maintain considerable relative proximity.

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Experimental procedures

Materials. All ligands (Figure 2) are named with a prefix “XY”, according to their class, where X = M or B depending on whether the ligand is monofunctional or bifunctional, respectively, and Y = T or N depending on whether the headgroup is based on tryptophan or naphthalene, respectively. Ligands **MT1** (L-tryptophan), **MT2** (L-tryptophan methyl-ester, hydrochloride salt), **MT4** (tryptamine), **MT6** (5-methoxy-*N*-acetyl tryptamine) were obtained commercially and were used without further purification.

Ligand Synthesis - Modifications to ligands **MT2** and **MT4** were performed using

the following protocol, where the term 'acid chloride' refers to the acetyl chloride for acetylation (**MT2**→**MT3**, **MT4**→**MT5**) or the benzoyl chloride for benzylation (**MT2**→**MT7**) reactions. 1 mmol of the free amine (**MT2** or **MT4**) was dissolved in dry dichloromethane (DCM) with two equivalents of triethylamine (Et₃N); one equivalent of the acid chloride was then added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 1 hour. The organic phase was washed in 1 M HCl, washed to neutral with H₂O, and dried over anhydrous sodium sulphate (Na₂SO₄). Where necessary, further purification was achieved by silica gel chromatography in 9:1 ethyl-acetate:petroleum-ether solution. The products were re-crystallised by slow diffusion of petroleum ether into DCM.

The synthesis for bifunctional ligands **BT1** and **BT2** involved the coupling of two **MT2** moieties by a di-acid chloride linker molecule using a method analogous to the *N*-modifications described above. The acid chlorides corresponding to the di-acid linkers (Figure 2B) were formed by activation of the di-acid with oxalyl chloride, using DCM as solvent and dimethylformamide (DMF) as a catalyst. 2mmol of **MT2** was dissolved in dry DCM with two equivalents of Et₃N. 0.5 equivalents of the acid chloride was added stepwise at 0 °C. The reaction was left stirring at room temperature for 12 hours and the products isolated as above.

The method used for the synthesis of the bifunctional naphthalene sulphonamide ligands is a modification of a previously published protocol [42]. Ligands **BN1** and **BN2** (Figure 2C) were synthesised by linking two 1-naphthalene sulphonylchloride moieties with *p*-xylylenediamine or *m*-xylylenediamine, respectively. 1 mmol of the

xylylenediamine was dissolved in 7.5 ml dry DCM and three equivalents of Et₃N added under nitrogen (N₂). 2 mmol of 1-naphthalene sulphonylchloride was dissolved in an equivalent volume of dry DCM and added dropwise to the xylylenediamine/Et₃N mixture, at room temperature under N₂. The reaction was left stirring at room temperature overnight under N₂. The reaction mixture was then washed in 1 M sodium hydroxide (aqueous) and the organic layer removed. The aqueous layer was further washed twice with dry DCM. All organic washes were pooled and dried over anhydrous Na₂SO₄. The products were re-crystallised by slow diffusion of hexane into DCM.

The characterisation of each new compound by NMR and elemental analysis is provided in the supplementary material. Stock solutions of the ligand samples were prepared by dissolution of recrystallised material in water, MeOH or DMSO as appropriate.

CaM Preparation. Protein samples were prepared from lyophilised recombinant mammalian CaM [43-45] by dissolution in 50 mM KCl, 10 mM CaCl₂, 0.5 mM NaN₃ at pH 6.0. Protein concentrations were quantified by titration with Ca²⁺ monitored by ¹H NMR. All experiments were carried out at 298K.

NMR Spectroscopy. Spectra were acquired using Bruker AMX-500 or DRX-500 spectrometers. Spectral widths were 12500 Hz and relaxation delays were typically 0.7 s for protein spectra and 3 s for ligand spectra. Solvent suppression was achieved using presaturation. PGSE experiments [32] included a composite 180°

pulse to refocus the magnetisation in the centre of a delay between two identical gradients. Sine shaped gradient pulses of 8 ms duration were used, with a centre to centre separation of 10.7 ms. Experiments were acquired at 0 % and 100 % gradient strength in order to obtain diffusion-edited spectra [46]. All experiments were acquired with a 2.5 ms pre-acquisition Hahn echo [47], and the water resonance removed with a convolution difference filter using FELIX (Accelrys, San Diego).

Fluorescence Spectroscopy. Experiments were performed using HITACHI-4500 and Shimadzu RF-5301 fluorescence spectrophotometers. For the peptide mimic ligands **MT2** and **BT2**, fluorescence emissions between 300 and 400 nm on excitation at 295 nm (**MT2**) or between 335 and 550 nm on excitation at 320 nm (**BT2**) were monitored for titrations of 0.5 mM CaM in KCl/CaCl₂ into 5 μM ligand in 10 % MeOH. The conformation of CaM is not significantly affected by the addition of 10% MeOH or 10% DMSO (see below), as determined by measuring ¹H,¹⁵H HSQC NMR spectra as a function of solvent composition (data not shown). For ligands **BN1** and **BN2**, titrations of 0.01 mM CaM into 1 μM ligand in 0.1 or 10% DMSO were monitored between 525 - 575nm with excitation at 275 nm. Slit widths were 2.5 nm and 5 nm for excitation and emission, respectively. For each ligand, control dilutions of ligand with KCl/CaCl₂ solution and of CaM into KCl/CaCl₂ solution were performed under the same conditions. The change in fluorescence intensity at 350 nm (**MT2**), 460 nm (**BT2**) or 550 nm (**BN1**, **BN2**) was used in data fitting to determine the dissociation constants for the interactions.

Determination of Dissociation Constants. Dissociation constants were derived by monitoring chemical shift changes of CaM resonances on addition of increasing ligand concentrations, or ligand fluorescence intensity changes on addition of increasing CaM concentrations. Data fitting was performed using n:1 stoichiometry where n sites are equivalent and independent. Interaction stoichiometries were determined by the model best fitting the data (minimum χ^2 value), both when fitting with integral values for n and when including n as a variable. The fit of the fluorescence data included a slope to accommodate a linear dependence of the fluorescence on increasing CaM concentration, observed in the control dilutions outlined above.

Isothermal Titration Calorimetry. The enthalpy associated with CaM binding was determined by isothermal titration calorimetry for **MT2** and **BT1**. Experiments were performed on a VP-ITC titration calorimeter (Microcal Inc., Northampton MA). Experiments were performed at least twice. Since **BT1** is not soluble at high concentration, dilution of the CaM complex was used to measure the enthalpy of dissociation of the complex. Lyophilized apoCaM was dissolved in unbuffered 100 mM KCl, 4 mM CaCl₂, 0.5 mM NaN₃ and dialysed overnight against the same solution. **MT2** or **BT1** was dissolved in methanol and added to the stock protein solution (0.4-0.5mM) to a final concentration of 210 μ M for **BT1** and 1.4 mM for **MT2**, and the final methanol content was adjusted to be 5% (v/v). All solutions were degassed for one minute to minimise evaporation of the methanol. The (**MT2**)₂:CaM or **BT1**:CaM complex was placed in the syringe. The solution placed in the cell was that used above for the dialysis of CaM, supplemented with 5% methanol, i.e. it is identical to the contents of the syringe except for the ligand:CaM complex. Aliquots

(10-15 μl for **MT2** and 15-20 μL for **BT1**) of the ligand:CaM complexes were added to the cell, and the data were fit to a dissociation isotherm to obtain the stoichiometry (n), the enthalpy of binding (ΔH) and the dissociation constant (K_d). The enthalpy associated with dilution of CaM or ligand solutions was also measured by injecting pure ligand and pure CaM solutions separately into the cell, but the effects were small compared with dilution of the complexes. The experiments were repeated at 8, 16, 25 and 32 $^{\circ}\text{C}$, and the temperature dependence of the enthalpy changes were used to obtain the values of ΔC_p . The high dissociation constant of the monofunctional ligand **MT1** resulted in relatively small heat effects, and the data for this ligand are therefore subject to a significantly larger error than for the strong binding ligand **BT1**.

SAXS measurements. Distance distribution functions were derived as described previously [48]. Protein concentrations exceeded the minimum dissociation constants of the chosen ligand by at least one order of magnitude.

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TABLE 1

Dissociation constants and stoichiometries for interactions between CaM and representative monofunctional and bifunctional ligands.

Ligand	K_d (μM)	Stoichiometry (ligand:CaM)	Method of K_d determination
MT2	400 ± 100	2	NMR, fluorescence
MT3	150 ± 60	2	NMR
MT7	60 ± 25	2	NMR
BT1	~ 10	1	NMR
BT2	9 ± 6	1	Fluorescence
BN1	0.07 ± 0.05	1	Fluorescence
BN2	0.01 ± 0.008	1	Fluorescence

TABLE 2

Thermodynamic parameters for monofunctional and bifunctional ligand binding to CaM measured by ITC at 25 °C.

	MT2	BT1
	1:1 monofunctional complex	1:1 bifunctional complex
K_d (μM)	600 ± 100	8 ± 1
ΔG (kJ mol^{-1})	-18.3 ± 0.5	-28.6 ± 0.5
ΔH (kJ mol^{-1})	-35 ± 3	-64 ± 1
$T\Delta S$ ($\text{kJ mol}^{-1} \text{K}^{-1}$)	-16 ± 3	-36 ± 1
ΔC_p ($\text{J mol}^{-1} \text{K}^{-1}$)	-1700 ± 900	-2600 ± 700

Figures

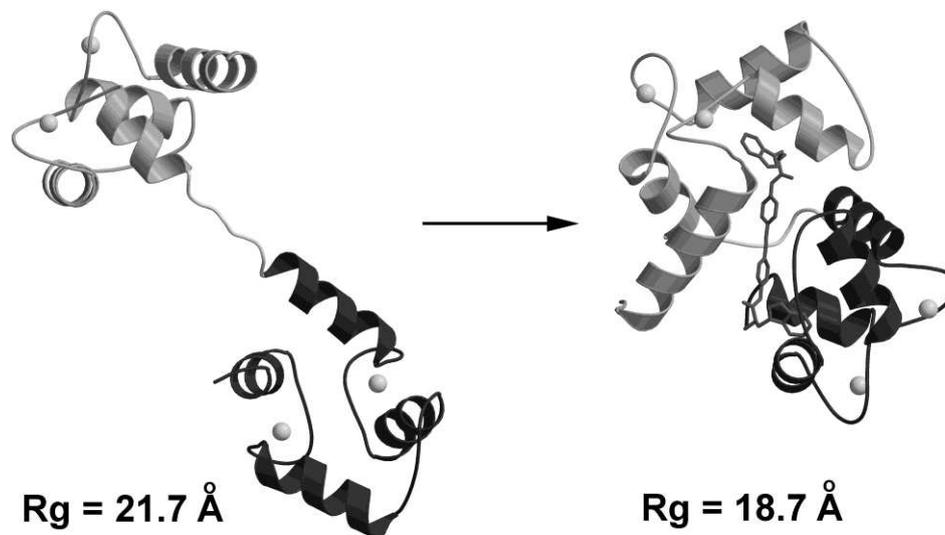


Figure 1. A schematic representation of the closure of CaM in response to a bifunctional ligand. Calcium loaded CaM (left) displays motional independence of the two domains, each of which has a hydrophobic pocket capable of binding a tryptophan moiety. In order to accommodate the bifunctional ligands consisting of two linked tryptophan head groups (right), CaM is required to form a closed conformation. The models are based on the pdb entries 3cln (left) and 1c1l (right), with the bifunctional ligand inserted using MACROMODEL [49]. The picture was generated using MOLSCRIPT [50] and Raster3D [51]. Below each model is the radius of gyration for the state determined from small-angle X-ray scattering distribution functions (see Figure 5) recorded on both 0.5 mM CaM and a 0.5 mM 1:1 complex between CaM and ligand **BT1** (see Figure 2B).

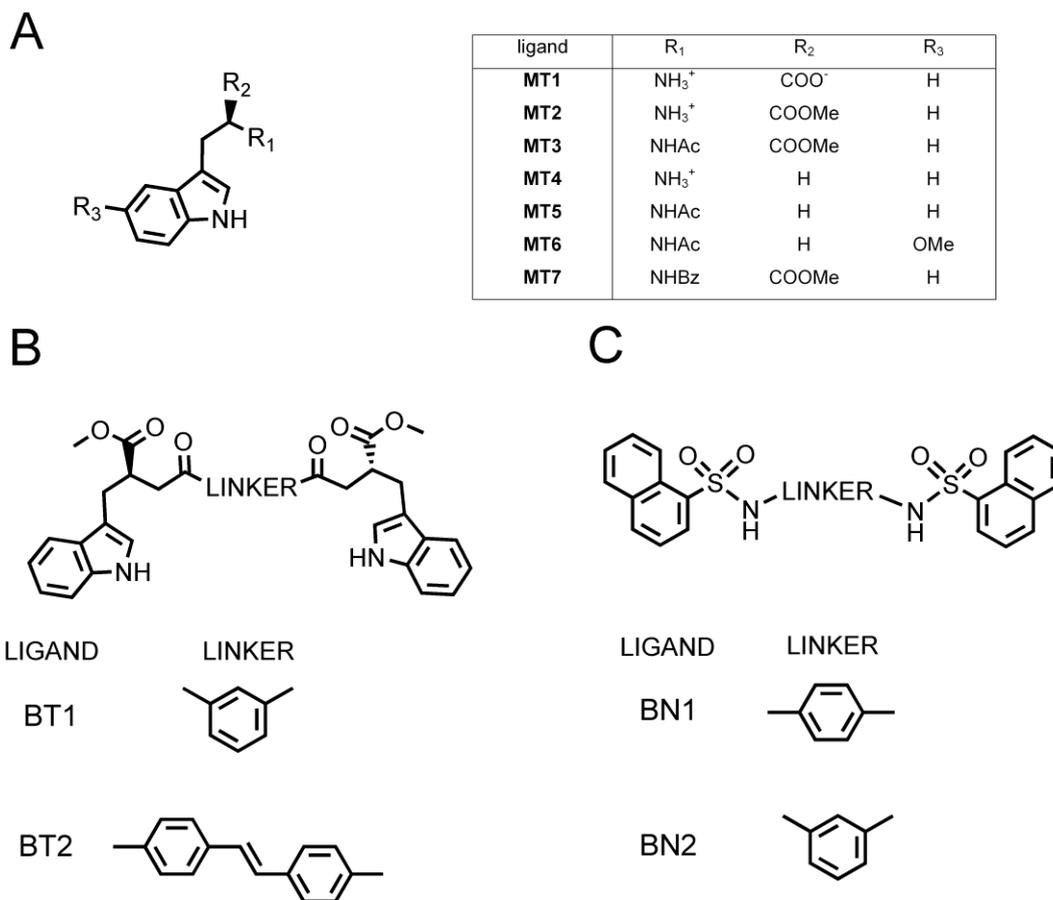


Figure 2. The structures of the ligands: **A**) monofunctional tryptophan ($R_2 = \text{COOR}$) and tryptamine ($R_2 = \text{H}$) ligands; **B**) the peptide-mimic bifunctional ligands, comprising two linked **MT2** moieties; **C**) the second generation bifunctional ligands, based on naphthalene sulphonamide headgroups.

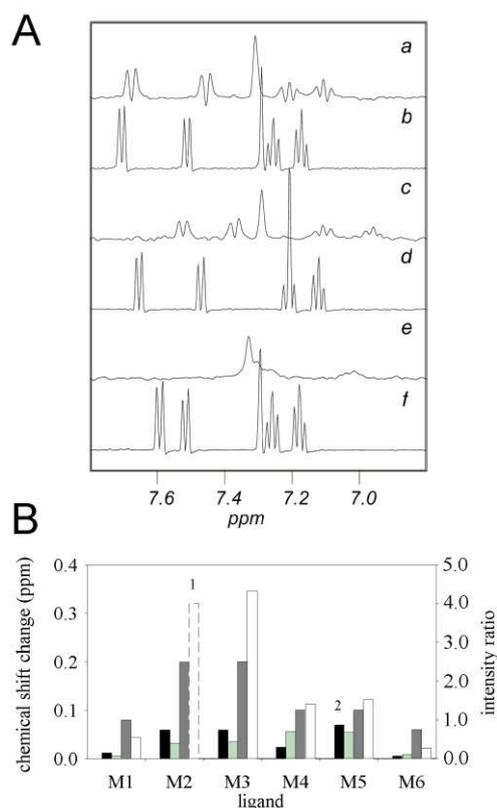


Figure 3. **A)** Aromatic region of diffusion-edited ^1H NMR spectra for 1 mM CaM samples containing 2 ligand equivalents (a,c,e), in comparison with free ligand spectra (b,d,f) for ligands **MT1** (a,b) **MT4** (c,d) and **MT2** (e,f). Free ligand spectra were acquired using 5 mM samples in D_2O (**MT1**, **MT2**) or 20 % $\text{d}_3\text{-MeOH}$ (**MT4**). **B)** Summary of NMR probes indicative of interaction between CaM and ligands **MT1** - **MT6**. For each measurement, larger bars indicate higher affinity binding. Protein shifts (black bars) and ligand indole NH shifts (lightgrey bars) were measured from initial - final chemical shifts in the titration of 5 aliquots of 0.5 equivalents of ligand into 1 mM CaM. Diffusion-edited spectra were derived from experiments acquired at 0% and 100% (50W) gradient strength. The intensity of the diffusion-edited spectrum is given relative to the intensity of the PGSE spectrum with 0 % gradient power (darkgrey bars, right vertical scale). The average shift changes for aromatic ligand resonances (white bars) were measured from the difference

between the diffusion-edited spectrum and the free ligand spectrum for identifiable signals. ¹ Average ligand shifts are lower limits for **MT2**, due to very low intensity signals. ² Estimated maximum value, since protein I27 and ligand indole NH resonances converge on increasing ligand:CaM ratios.

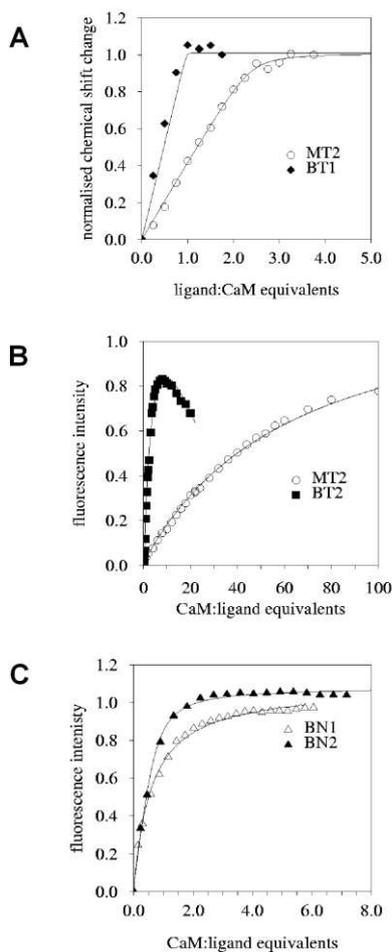


Figure 4. Plots showing the change in observed signal for representative titrations, together with the lines of best fit obtained by least-squares fitting of the data. **A)** Normalised NMR chemical shift changes for CaM I27 amide resonance on addition of ligands **MT2** (open circles) or **BT1** (filled diamonds). **B)** Normalised fluorescence changes observed on addition of CaM to peptide-mimic ligands **MT2** (open circles) or **BT2** (filled squares), monitored at 350nm and 460nm, respectively. For consistency in reported fluorescence changes, the observed values for the **BT2** experiment (decreasing fluorescence) are inverted relative to those for **MT2** (increasing fluorescence). **C)** Normalised fluorescence changes observed on addition of CaM to ligands **BN1** (open triangles) and **BN2** (filled triangles) monitored at 550nm.

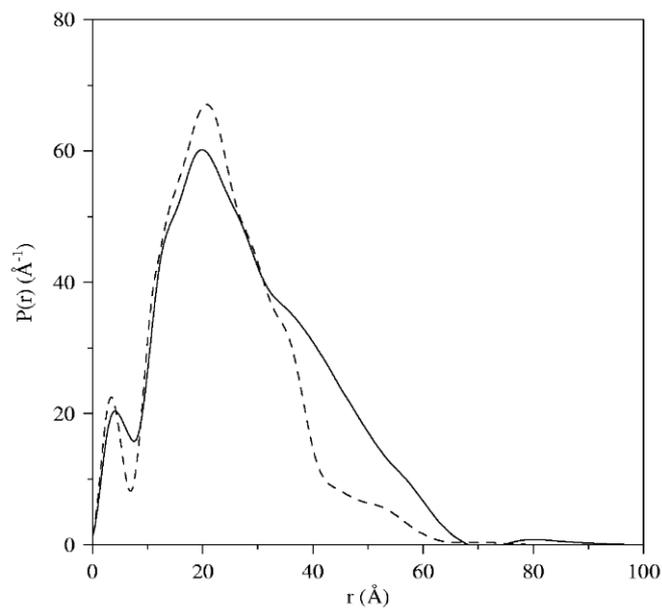


Figure 5. A comparison of the distance distribution $P(r)$ functions for calcium-loaded CaM (solid line) and a 1:1 complex of **BT1** and calcium-loaded CaM (broken line), illustrating the compaction that occurs on binding of the bifunctional ligands. The radius of gyration falls from 21.7 Å to 18.7 Å on formation of a 1:1 complex.