High-affinity formation of a 2:1 complex between gramicidin S and calmodulin

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Two molecules of gramicidin S, a very rigid cyclic decapeptide rich in β -sheet structure, can bind in a Ca^{2+} -dependent way to a calmodulin molecule in the presence as well as in the absence of 4 M-urea. The flow-microcalorimetric titration of 25 μM -calmodulin with gramicidin S at 25 °C is endothermic for 21.3 kJ $\cdot$ mol $^{-1}$; the enthalpy change is strictly linear up to a ratio of 2, indicating that the affinity constant for binding of the second gramicidin S is at least 10^7 M^{-1} . In 4 M-urea the peptide quantitatively displaces seminalplasmin from calmodulin, as monitored by tryptophan fluorescence. An iterative data treatment of these competition experiments revealed strong positive co-operativity with $K_1 < 5 \times 10^5 \text{ M}^{-1}$ and $K_1 \cdot K_2 = 2.8 \times 10^{12} \text{ M}^{-2}$. A competition assay with the use of immobilized melittin enabled us to monitor separately the binding of the second gramicidin S molecule: the K_2 value is $1.9 \times 10^7 \text{ M}^{-1}$. By complementarity, the K_1 value is $1.5 \times 10^5 \text{ M}^{-1}$. In the absence of urea the seminalplasmin displacement is incomplete: the data analysis shows optimal fitting with $K_1 < 2 \times 10^4 \text{ M}^{-1}$ and $K_1 \cdot K_2 = 3.2 \times 10^{11} \text{ M}^{-2}$ and reveals that the mixed complex (calmodulin–seminalplasmin–gramicidin S) is quite stable and is even not fully displaced from calmodulin at high concentrations of gramicidin S. The activation of bovine brain phosphodiesterase by calmodulin is not impaired up to 0.2 μM -gramicidin S. According to our model the ternary complex enzyme–calmodulin–gramicidin is relatively important and displays the same activity as the binary complex enzyme–calmodulin. Gramicidin S also displaces melittin from calmodulin synergistically, as monitored by c.d. Our studies with gramicidin S reveal the importance of multipoint attachments in interactions involving calmodulin and confirm the heterotropic co-operativity in the binding of calmodulin antagonists first demonstrated by Johnson [(1983) *Biochem. Biophys. Res. Commun.* **112**, 787–793].

INTRODUCTION

Calmodulin (CaM) is an important sensor protein of intracellular free Ca^{2+} transients and undergoes Ca^{2+} -dependent conformational changes leading to interaction with and activation of numerous target enzymes [1]. As part of the activation mechanism, two hydrophobic domains on CaM are exposed when Ca^{2+} binds [2,3]. The latter sites are thought to be the regions when CaM interacts not only with its target enzymes but also with amphiphilic drugs [4,5]. Covalent-labelling [3,6] and fragment studies [5,7,8] indicate that one drug-binding site is present in each half of the dumb-bell-shaped CaM molecule. CaM forms a Ca^{2+} -dependent 2:1 complex with hydrophobic drugs (for review see [9]) and with certain peptides, such as β -endorphin [10,11]. Generally, the affinity is around 10^6 M^{-1} [12], i.e. three orders lower than the affinity of natural target enzymes for CaM. Johnson and co-workers [13,14] demonstrated that strong positive co-operativity can exist between the two drug-binding sites. For instance, binding of 2 mol of felodipine/mol displays a Hill coefficient of 2 (homotropic co-operativity); similarly, binding of certain 'potentiating drugs' (prenylamine, R24571) to one site increases the affinity of the remaining site for felodipine 25-fold (heterotropic co-operativity). It should be noted that in CaM these phenomena of homotropic and

heterotropic co-operativity are strongly dependent on the nature of the drug (for review see [12]) and that the integrity of CaM is obligatory [5].

Interestingly, a category of natural peptides, including melittin [15], mastoparans [16] and seminalplasmin (SP) [17], of synthetic model peptides [18,19] and peptides originating from target enzymes [20,21] form 1:1 high-affinity ($K_D = 10^{-9} \text{ M}$) complexes with CaM. They all become α -helical upon interaction [18–23]. These data, the recent reports by Steiner and coworkers [24,25] and the data of Seeholzer *et al.* [26] strongly suggest to us that the high-affinity α -helical peptides are capable of forming a bridge between the two hydrophobic sites in the *N*- and *C*-terminal halves of the CaM molecule, along the central α -helix of CaM. These two- or multi-point attachments would logically lead to a strong synergistic increase of the affinity as compared with compounds that form 2:1 complexes with CaM.

Gramicidin S (GS) is a cyclic decapeptide antibiotic from *Bacillus brevis* with the sequence:

Its conformation can be described as a very rigid two-stranded antiparallel β -sheet with β -bends at the

Abbreviations used: CaM, calmodulin; GS, gramicidin S; SP, seminalplasmin; PDE, cyclic nucleotide phosphodiesterase; Melex, melittin–Sephacrose 4B.

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D-Phe-Pro sequences at both ends ([27], and references cited therein). Molecular modelling indicates that it has a compact hydrophobic face flanked by basic residues (W. F. DeGrado, personal communication). Since amphiphilicity is considered to be of importance in the interaction of peptides with CaM [18,19,23], and GS is a rather simple molecule with resolved tertiary structure, we have chosen to study the interaction of GS with CaM.

MATERIALS AND METHODS

Materials

GS was purchased from Sigma Chemical Co. and further purified by gel filtration on Sephadex G-25 in 10 mM-ammonium acetate buffer, pH 5.0, containing 30% (v/v) ethanol. Bovine SP was affinity-purified by the method of Comte *et al.* [17]. Melittin was prepared by the method of Maulet *et al.* [28]. Bovine brain CaM was purified by affinity chromatography as described by Gopalakrishna & Anderson [29]. The purity of these polypeptides was checked by h.p.l.c. on a μ Bondapak C₁₈ column (Waters Associates). Mono[³H]acetyl-CaM and melittin-Sepharose 4B (Melex) were prepared as previously described [18]. Protein and peptide concentrations were determined spectrophotometrically by using the following molar absorption coefficients: $\epsilon_{278} = 3020 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for metal-ion-free CaM, $\epsilon_{258} = 392 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for GS, $\epsilon_{280} = 5500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for melittin and $\epsilon_{280} = 6970 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for SP. CaM-deficient bovine brain phosphodiesterase (PDE) was prepared by the method of Watterson *et al.* [30].

Experiments of cross-linking between CaM and peptides

These were carried out at 4 °C in 0.35 ml of 135 mM-NaCl/1 mM-CaCl₂/4 M-urea/60 mM-Tes/NaOH buffer, pH 7.0, containing 7 μ M-CaM in the presence or in the absence of 67 μ M-GS and/or 3.9 μ M-melittin. The reaction was initiated by adding disuccinimidyl suberate (Pierce Chemical Co.) to a final concentration of 140 μ M. After 270 min of incubation the reaction was stopped by adding 1.1 mM-ethylenediamine. Samples were micro-dialysed against water and subjected to polyacrylamide-(11.25%)-disc-gel electrophoresis in the presence of SDS and EDTA (1 mM).

Equilibrium-dialysis experiments

Equilibrium dialysis experiments in order to determine the stoichiometry between CaM and GS were carried out at 24.0 °C in 100 mM-NaCl/0.5 mM-CaCl₂/20 mM-Tes/NaOH buffer, pH 7.0, in the presence or in the absence of 4 M-urea. A 1 ml solution containing 100 μ M-CaM and 250 μ M-GS was dialysed in a Visking bag (M_r cut-off approx. 12000) for 120 h against 120 ml of the dialysis buffer containing 10 μ M-GS with one buffer change after 24 h. The concentration of GS in the diffusate was monitored by h.p.l.c. as described above. The amount of GS bound to CaM in the dialysis residue was determined by amino acid analysis after standard hydrolysis of three samples in 6 M-HCl for 48 h. Amino acid analyses were carried out on a Beckman 119 CL amino acid analyser under conditions where ornithine was eluted simultaneously with the histidine peak. After comparison of the amino acid content with that of CaM, the increased amounts of the amino acids proline, valine, leucine and phenylalanine

enabled calculation of the ratio of GS present in the samples.

Microcalorimetry

Enthalpy changes were determined in an LKB 2277 flow microcalorimeter at 25 ± 0.1 °C as described previously [31]. The stock solution of CaM (50 μ M) was equilibrated in the reaction medium, 150 mM-NaCl/1 mM-CaCl₂/50 mM-Pipes/NaOH buffer, pH 7.0, or 150 mM-NaCl/1 mM-CaCl₂/50 mM-Tris/HCl buffer, pH 7.0, by equilibrium gel filtration [28]. The concentrated (approx. 3 mM) stock solution of GS in ethanol/water (3:7, v/v) was diluted with the reaction buffer to the indicated concentrations. The experimental procedure encompasses correction for the dilution enthalpy of ethanol. Proton release upon complex-formation was calculated from the microcalorimetric experiments as described previously [31,32].

Competitive fluorescence titrations

These were carried out at room temperature on a Baird spectrofluorimeter with the excitation wavelength at 290 nm and the emission fixed at 330 nm. To equimolar mixtures of CaM and SP in 0.1 M-NaCl/0.5 mM-CaCl₂/20 mM-Tes/NaOH buffer, pH 7.0, with or without 4 M-urea, increasing amounts of GS were added up to a final concentration of 500 μ M. At the indicated wavelengths, the complex-formation between CaM and SP leads to a 2-fold enhancement of the fluorescence of the unique tryptophan residue in SP [17]. The addition of GS leads to full (urea present) or partial (urea absent) reversal of the fluorescence enhancement. Control experiments where GS was added to CaM or to SP alone did not lead to any fluorescence change at the indicated wavelengths. Displacement curves were obtained by expressing the fluorescence decrease (relative to the fluorescence of SP in the presence or in the absence of 1 equivalent of CaM) as a function of added GS.

Competitive Melex and PDE assays

These were carried out as described previously [15,18].

C.d. titrations

These were carried out at 20 °C on a Jasco J-20 spectropolarimeter with a 0.2 cm-light-path quartz cuvette in 100 mM-NaCl/0.5 mM-CaCl₂/4 M-urea/20 mM-Tes/NaOH buffer, pH 7.0. To a mixture of 5 μ M-CaM and 5 μ M-melittin, which displays 25% more ellipticity at 222 nm than does CaM alone [22], increasing amounts of GS were added up to a final concentration of 75 μ M. In control experiments similar increments of GS were added to 5 μ M-CaM alone or to the buffer. GS itself shows an ellipticity signal at 222 nm, but complex-formation with CaM does not affect its ellipticity intensity, nor that of CaM (see the Results section), and therefore its contribution to the signal at 222 nm can be corrected for. Displacement of melittin from CaM by GS was accompanied by a decrease in ellipticity at 222 nm, indicating that upon dissociation melittin loses its α -helical conformation [22].

RESULTS

Ca²⁺-dependent cross-linking between CaM and GS

Direct evidence of Ca²⁺-dependent complex-formation between GS and CaM was obtained by electrophoresis in

Fig. 1. Demonstration by covalent cross-linking of a complex between GS and CaM

The cross-linking and electrophoreses were carried out as described in the Materials and methods section. All lanes contain 11 μg of CaM. In the first five lanes, CaM samples were submitted to the cross-linker disuccinimidyl suberate (+DSS) in the presence of 1 mM- CaCl_2 (+) or of 1 mM-EDTA (–) and of the peptides as indicated (ME, melittin). The faint band below the uncomplexed CaM band is very probably internally cross-linked CaM [11].

Fig. 2. Enthalpy titration of GS binding to CaM in the presence of Ca^{2+}

The CaM concentration after mixing was 25 μM . Experiments were carried out in 150 mM- NaCl /1 mM- CaCl_2 /50 mM-Pipes/NaOH buffer, pH 7.0, (●) or 150 mM- NaCl /1 mM- CaCl_2 /50 mM-Tris/HCl buffer, pH 7.0 (○).

SDS-containing medium after covalent cross-linking with disuccinimidyl suberate (Fig. 1). Whereas CaM alone migrated with a relative mobility (R_F) of 0.67, two new bands appeared when it was cross-linked with GS: a major one with an R_F of 0.64 and a minor one with an R_F of 0.62. Under identical conditions the cross-linked CaM-melittin complex showed an R_F of 0.60. Taking the latter (M_r 19630) and unmodified CaM (M_r 16780) as markers, the plot of $\log M_r$ versus relative mobility (not shown) indicates that the major band corresponds to a CaM-GS₁ complex (M_r 17930) and the minor one to the CaM-GS₂ complex (M_r 19070). None of these complexes was formed in the presence of 1 mM-EDTA, indicating that complex-formation is Ca^{2+} -dependent (second lane from left). In the presence of 7 μM -CaM and both

Fig. 3. Tryptophan emission fluorescence spectra of SP in the presence of CaM and GS

The spectra were recorded at 25 °C with the excitation wavelength at 290 nm. Measurements were made in 100 mM- NaCl /0.5 mM- CaCl_2 /4 M-urea/20 mM-Tes/NaOH buffer, pH 7.0. The spectra were corrected for the contribution of CaM. —, 10 μM -SP; ----, 10 μM -SP + 10 μM -CaM; - · - · -, 10 μM -SP + 10 μM -CaM + 300 μM -GS.

67 μM -GS and 3.9 μM -melittin, both the CaM-GS₁ and CaM-melittin covalent complexes are formed without formation of a ternary adduct. The intensity of the CaM-GS₁ complex is diminished compared with that of the same band in the absence of melittin. These data indicate, first, that melittin and GS are competitors for CaM, and, secondly, that GS has less affinity for CaM than melittin does.

Stoichiometry of the CaM-GS complex

Equilibrium dialysis indicated that at 10 μM -GS 1 mol of CaM binds 2.04 ± 0.25 mol of GS in the absence and 1.92 ± 0.30 mol in the presence of 4 M-urea. A 2:1 stoichiometry was further confirmed by enthalpy titrations in Pipes and Tris buffers (see below). The fact that the covalent cross-linking experiment revealed mostly the 1:1 complex indicates that, although CaM bound 2 mol of GS/mol, one binding site shows a more favourable configuration for covalent cross-linking with disuccinimidyl suberate.

Enthalpy titration of the CaM-GS interaction

The evolution of heat absorption upon increasing the ratio of GS per CaM in the presence of saturating [Ca^{2+}] is strictly linear up to 2 mol of GS/mol of CaM (Fig. 2). The sharp inflexion in the profile upon titration of 25 μM -CaM (after mixing) points to a rather high affinity ($K \geq 10^7 \text{ M}^{-1}$), at least for the binding of the second GS molecule. Since proton release or uptake can lead to an erroneous estimation of the standard free enthalpy change of a binding process [31], we measured the thermal effects at pH 7.0 in two buffers of very different

Fig. 4. Competitive displacement of SP from CaM by GS as monitored by tryptophan fluorescence at 330 nm

The estimated error on the measurements is 2.5%. (a) Buffer was as in Fig. 3, including 4 M-urea. The samples contained equimolar concentrations of 3 μM- (●), 10 μM- (×) or 20 μM-CaM-SP (○). The continuous lines are the computed displacement curves obtained according to Scheme 1 by using the data treatment explained in the Results section. The following formation constants were used: $K_{SP} = 6.7 \times 10^8 \text{ M}^{-1}$; $K_{GS_1} = 1.5 \times 10^5 \text{ M}^{-1}$; $K_{GS_1} \cdot K_{GS_2} = 2.8 \times 10^{12} \text{ M}^{-2}$; $K'_{SP} \cdot K'_{GS_1} = 1.8 \times 10^{12} \text{ M}^{-2}$. The broken line represents the appearance of the mixed complex SP-CaM-GS during the titration of 10 μM-CaM-SP (in percentage of total SP). (b) Similar displacement experiments in the absence of urea starting with 3 μM of CaM-SP complex. The continuous line was obtained as above by using the following formation constants: $K_{SP} = 6.7 \times 10^8 \text{ M}^{-1}$; $K_{GS_1} = 10^4 \text{ M}^{-1}$; $K_{GS_1} \cdot K_{GS_2} = 3.2 \times 10^{11} \text{ M}^{-2}$; $K'_{SP} \cdot K'_{GS_1} = 2.0 \times 10^{13} \text{ M}^{-2}$. The broken line represents the appearance of the mixed complex SP-CaM-GS during the titration.

protonation enthalpy. Fig. 2 shows that the ΔH^0 for binding of 2 mol of GS/mol amounts to 19.68 and 14.63 kJ·mol⁻¹ in Pipes and Tris buffer respectively. Applying a previously established rule [32], we calculated that 0.14 mol of H⁺ is released/mol of CaM upon binding of 2 mol of GS and that in this process the real (i.e. buffer-independent) ΔH^0 amounts to 21.28 kJ·mol⁻¹. Microcalorimetric experiments in the presence of 4 M-urea under otherwise identical conditions did not allow us to establish valuable enthalpy titration curves.

Competition between GS and SP for CaM

GS dissociates the high-affinity 1:1 complex between CaM and SP, which can be studied quantitatively by the tryptophan fluorescence of the latter peptide as mentioned in the Materials and methods section. GS influences neither the tryptophan fluorescence of SP nor the tyrosine fluorescence of CaM. This is also confirmed by the fact that complex-formation between CaM and GS does not influence at all the near-u.v. c.d. spectrum of CaM in the region 270–300 nm (results not shown). Fig. 3. shows that in the presence of 4 M-urea GS quantitatively reverses the 2-fold tryptophan fluorescence enhancement as well as the blue-shift that accompanies the SP-CaM interaction [17]. The displacement curves of Fig. 4 with 3 μM-, 10 μM- and 20 μM-CaM-SP complex

Scheme 1

It should be noted that the value of the formation constant $K'_{SP} \cdot K'_{GS_1}$ can be equal (independent SP and GS sites), smaller (competitive sites) or greater (synergistic sites) than the product of K_{SP} and K_{GS_1} .

were obtained as indicated in the Materials and methods section. They could be analysed quantitatively by assuming the equilibria (1)–(4) shown in Scheme 1.

A priori information is available on only two of the binding constants of Scheme 1: K_{SP} is equal to $6.7 \times 10^8 \text{ M}^{-1}$ [17] and K_{GS_2} must be higher than 10^6 M^{-1} (from equilibrium-dialysis data above). To obtain the other affinity constants the data of Fig. 4 were analysed as follows. Giving arbitrary values to these constants, we calculated the concentration of the different species of Scheme 1 with the computer program of Perrin & Sayce [33] for multi-ligand mixtures. We assume that the tryptophan fluorescence of SP in the mixed complex SP-CaM-GS is enhanced and blue-shifted to the same extent as in CaM-SP [17]. On these premises the sum of the concentrations of CaM-SP and SP-CaM-GS as a function of added GS represents the displacement curves of Fig. 4. Iteratively the formation constants of the equilibria (2), (3) and (4) were adapted to provide an optimal fitting with the experimental displacement curves. The three experiments performed in 4 M-urea at different concentrations of initial complex yielded good fits with $K_{GS_1} < 5 \times 10^5 \text{ M}^{-1}$, $K_{GS_1} \cdot K_{GS_2} = 2.8 \times 10^{12} \text{ M}^{-2}$ and $K'_{SP} \cdot K'_{GS_1} = 1.8 \times 10^{12} \text{ M}^{-2}$. Comparison of theoretical curves using different sets of binding constants with experimental data revealed that the values for the predominant formation constants are accurate to within $\pm 20\%$. The K_{GS_1} value cannot be determined precisely since $[\text{CaM-GS}_1]$ is very low during the titration and therefore the second equilibrium of Scheme 1 has little or no effect on the overall exchange reaction. Anyway, binding of the second GS molecule displays an affinity constant at least one order higher than binding of the first, thus revealing a strong positive co-operativity in the binding of GS. Another interesting information, evidenced by the above data treatment, is that $K'_{SP} \cdot K'_{GS_1}$ is at least 100-fold smaller than $K_{SP} \cdot K_{GS_1}$; thus the mixed complex SP-CaM-GS is never abundant during the titration in the presence of urea. For instance the concentration of the mixed complex never exceeds 5% of all complexes during the titration of 10 μM of complex (Fig. 4a).

In the absence of urea the tryptophan fluorescence decrease curve describing displacement of 3 μM-CaM-SP complex by GS levels off at a submaximal plateau (Fig. 4b), although binding of the second GS displays an affinity constant higher than 10^7 M^{-1} , as can be inferred from the microcalorimetric experiments (Fig. 2). The

Scheme 2

analysis of the curves as described above with a K_{SP} value as in the presence of urea [17] indicates that in this case the formation constants of the GS-CaM complexes of Scheme 1 are about one order of magnitude lower than those in urea: more interestingly, the fourth constant is 10-fold higher, i.e. $K'_{\text{SP}} \cdot K'_{\text{GS}_1} = 2 \times 10^{13} \text{ M}^{-2}$, and nearly equal to $K_{\text{SP}} \cdot K_{\text{GS}_1}$. This means that in the absence of urea the formation of the mixed complex SP-CaM-GS (with full tryptophan fluorescence enhancement and blue-shift) is favoured over that of CaM-GS₂ and can amount to 41% of all complexes during the titration (Fig. 4b).

Displacement of CaM from Melex by GS in 4 M-urea

The competitive Melex assay was used previously to determine the affinity in 1:1 complexes between CaM and different peptides [17–19]. In the light of the formation of 2:1 complexes between GS and CaM, the displacement of CaM monitored from Melex by GS is expected to occur upon binding of the second GS to CaM as predicted by Scheme 2. Since only step 2 is monitored, the Melex assay constitutes an independent way of determining K_{GS_2} . Fig. 5 shows a strict parallelism of the curves describing displacement by GS or by free melittin. The constant, calculated as described previously [18], amounts to $1.9 \times 10^7 \pm 0.2 \times 10^7 \text{ M}^{-1}$ in three independent experiments.

Competitive enzymic assay with PDE

The PDE assay was previously used as a tool for determining the affinity of model peptides for CaM

Fig. 5. Displacement of Melex-bound [³H]CaM by GS as compared with melittin

The incubation mixture contained 5.0 μM immobilized melittin units, 150 nM-CaM, trace amounts of mono-[³H]acetyl-CaM and increasing amounts of soluble melittin (x) or GS (●). The assay buffer was 135 mM-NaCl/1 mM-CaCl₂/60 mM-Tes/NaOH buffer, pH 7.0. The amount of Ca²⁺-sensitive complex between CaM and Melex (f_b) was determined as previously described [18]. The vertical bar corresponds to the zone of f_b where the determination of the K_D yields reproducible values.

Fig. 6. Influence of GS on the CaM-mediated activation of bovine brain PDE

The PDE activity (expressed as fraction of maximal activation) was measured at a constant CaM concentration corresponding to 60% of maximal activation and increasing concentrations of GS. The continuous curve was computed as in Fig. 4 by using the following formation constants: $K_{\text{PDE}} = 10^9 \text{ M}^{-1}$; $K_{\text{GS}_1} = 10^4 \text{ M}^{-1}$; $K_{\text{GS}_1} \cdot K_{\text{GS}_2} = 3.2 \times 10^{11} \text{ M}^{-2}$; $K'_{\text{PDE}} \cdot K'_{\text{GS}_1} = 8 \times 10^{12} \text{ M}^{-1}$.

[15,17–19]. Surprisingly, GS at 500 nM only very slightly shifts the curve describing the CaM-dependent activation of PDE to higher concentrations of activator (results not shown). A GS dose-response curve at a constant CaM concentration corresponding to 60% stimulation (Fig. 6) shows that inhibition becomes important at micromolar concentrations of GS. Since previous experiments demonstrated the high affinity of GS for CaM, we expected an important shift of the activation curve to higher concentrations of CaM. In the light of the importance of mixed complexes [reaction (4) in Scheme 1] in the absence of urea (Fig. 4b) and of the observation that CaM covalently modified in one of its hydrophobic sites can still bind and activate certain enzymes, we assume that in this assay too a ternary PDE-CaM-GS complex is formed and that the latter is as active as PDE-CaM. With computer simulation as indicated in the SP-displacement experiments, the curve of Fig. 6 could be reproduced with $K_{\text{PDE}} = 10^9 \text{ M}^{-1}$ [34], $K_{\text{GS}_1} \cdot K_{\text{GS}_2} = 3.2 \times 10^{11} \text{ M}^{-2}$ and $K'_{\text{PDE}} \cdot K'_{\text{GS}_1} = 8.0 \times 10^{12} \text{ M}^{-1}$. It should be noted that the latter value is close to the formation constant of the mixed SP-CaM-GS complex in the absence of urea.

C.d. properties of CaM-GS complexes and melittin displacement

A mixture of 10 μM -CaM with 10 μM -GS displays an ellipticity at 222 nm corresponding to the sum of that of CaM and GS alone (results not shown). This is further illustrated in Fig. 7: the ellipticity increase is directly proportional to the concentration of added GS, independently of the presence of CaM. These data indicate that complex-formation between CaM and GS alters neither the α -helical content of CaM nor the β -pleated sheet content of GS, which is not surprising given the strong conformational constraints of GS. The

Fig. 7. Competitive displacement of melittin from CaM by GS as monitored by far-u.v. c.d. in the presence (■ and ●) or in the absence (□ and ○) of 4 M-urea

Ellipticity (θ_{222}) titration of 5 μM -CaM-melittin (■ and □) or 5 μM -CaM (● and ○) with GS. The ellipticity increase due to GS alone in the presence (×) and in the absence (+) of urea is also indicated.

absence of an ellipticity change in this interaction allowed us to study the displacement of melittin by GS in the presence and in the absence of 4 M-urea. In 4 M-urea the c.d. titration of the CaM-melittin complex with increasing concentrations of GS results in a less than proportional increase of the ellipticity at 222 nm. It can therefore be assumed that GS displaces melittin competitively from CaM, and that the ellipticity decrease

results from the transition of free melittin from its α -helical to its random-coil conformation. In the absence of urea the ellipticity decrease is much less pronounced, which again indicates that the mixed complex melittin-CaM-GS is much more stable than in the presence of urea.

DISCUSSION

Together, the two displacement experiments in the presence of urea (the displacement of SP from CaM and of CaM from Melex) yield good estimates of the intrinsic association constants K_{GS_1} and K_{GS_2} and illustrate the strong positive homotropic co-operativity in the binding of GS to CaM. In the absence of urea the SP-displacement experiment (Fig. 4b) and PDE competitive assay also point to co-operative binding of GS to CaM with 10-fold lower association constants than in the presence of urea. The latter and the microcalorimetric data enable us to evaluate the nature of the driving forces of the interaction of GS with CaM. The overall formation constant $K_{\text{GS}_1} \cdot K_{\text{GS}_2}$ ($= 3.2 \times 10^{11} \text{ M}^{-2}$) corresponds to a ΔG^0 value of $-65.7 \text{ kJ} \cdot \text{mol}^{-1}$; with a ΔH^0 value of $21.3 \text{ kJ} \cdot \text{mol}^{-1}$ (Fig. 2) a ΔS^0 value of $293 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ can be calculated. Thus, as in the case of the interaction CaM-melittin with its ΔH^0 value of $30.3 \text{ kJ} \cdot \text{mol}^{-1}$ and its ΔS^0 value of $275 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ [32], the primary driving force is partial withdrawal of the non-polar groups from water. Given the strong positive co-operativity, no significant amounts of the intermediate species CaM-GS₁ are formed during the enthalpy titration in Fig. 2. This explains the strict linearity of the profile but also precludes that any conclusion can be drawn about the thermodynamics of the individual binding steps of GS.

With its homotropic co-operativity, the binding of GS resembles that of felodipine, although the formation constant of CaM-felodipine₂ is $2 \times 10^6 \text{ M}^{-1}$ [14], i.e. 2–3 orders lower than that of CaM-GS₂. The SP-displacement experiment in the absence of urea as well as the PDE assay indicate that binding of one molecule of GS/molecule does not decrease the affinity of SP or PDE

Scheme 3. Model of the displacement of a high-affinity α -helical peptide, here melittin (ME), from CaM by GS

The dumb-bell shape of CaM and the boomerang shapes of melittin and the GS dimer are taken from crystallographic studies on these compounds. The hydrophobic sites 1 and 2 in the N- and C-terminal halves of CaM are located arbitrarily not too far from the central α -helix. In the presence of 4 M-urea, but not in its absence, the intermediate ternary complex is unstable (see the Results section).

for CaM and vice versa, but eventually potentiates binding. Competition is observed at high GS concentrations only. Again this is reminiscent of the system felodipine/prenylamine [14]. The stability of a ternary complex SP-CaM-GS or PDE-CaM-GS in the absence of urea is not surprising in the light of the report by Newton *et al.* [35] that CaM labelled covalently with 1 mol of norchlorpromazine/mol binds with high affinity with at least six target enzymes, whereas the twice-labelled adduct is fully inactive in binding to enzymes. Thus GS is not just a competitive inhibitor of enzymes or high-affinity model peptides. However, for reasons presently not understood, the competitive nature of GS is much more dominant in the presence of 4 M-urea, suggesting that hydrogen bonds are important for the synergistic action between the two drug-binding sites.

The question arises whether the homotropic cooperativity in binding of GS to CaM is caused by multi-point attachment. It should be noted that GS crystallizes as a hydrated 2.15 nm-long dimer of the following composition: (GS)₂urea₁ [36]. This dimer is boomerang-shaped with a typical amphiphilic orientation of the positive and hydrophobic side-chain residues [27]. If GS also shows this configuration in the complex with CaM, the dimer would be able to span, and neutralize, the *N*- and *C*-terminal hydrophobic sites on CaM, mostly the same way as the three-turn-long α -helical peptides do. Additional stabilization by electrostatic bonds between the four ornithine residues and the carboxy groups in the central α -helix of CaM would lead to the observed high-affinity interaction.

The displacement curves of SP or melittin in the presence of CaM can be interpreted by the same model of multi-point attachment. Scheme 3 tentatively describes the displacement reaction assuming the simplest model, i.e. two-point attachment of melittin to CaM. In this Scheme the hydrophobic sites on CaM are numbered 1 and 2, and are supposed to have microscopic affinity constants of 10^4 – 10^6 M⁻¹ for any target. When melittin (or any other high-affinity α -helical peptide) binds, the overall affinity constant is much higher, owing to synergy of both the hydrophobic interactions in sites 1 and 2 and the electrostatic ones along the central α -helix of CaM. When GS competitively displaces melittin at one hydrophobic site a ternary complex is formed that is rather stable in the absence of urea, but is quickly followed by binding of a second GS molecule and dissociation of melittin from CaM in the presence of urea. As a consequence of dimer interactions in GS, the affinity constant of the latter is distinctly higher than in cases where two non-interacting CaM antagonists, such as phenothiazines, bind to CaM.

An interesting piece of information revealed in this study is that a quite-high-affinity complex (K_D approx. 50 nM) is formed between CaM and a peptide that undoubtedly is amphiphilic and positively charged but cannot exist as an α -helix. Apparently, our previously postulated rules about the minimal structural features required of a high-affinity ligand for CaM [18] are transgressed in the case of GS. Recently, this was also observed in the interaction of CaM with the inhibitory peptide 104–115 of troponin I, which also interacts with CaM without possessing or gaining any α -helical structure [37]. However, this peptide displays a low affinity for CaM (K_D approx. 1 μ M) [37]. Even if the insertion of the inhibitory peptide in whole troponin I

dramatically increases the affinity for troponin C as a result of multipoint attachment, this model seems not to be applicable to the case of CaM, where very short peptides excised from natural target enzymes display the same binding characteristics as the bulky enzymes themselves [20,21].

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