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Affinity purification of seminalplasmin and characterization of its interaction with calmodulin

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Bull seminalplasmin antagonizes with high potency and selectivity the activating effect of calmodulin on target enzymes [Gietzen & Galla (1985) Biochem. J. 230, 277–280]. In the present paper we establish that seminalplasmin forms a 1:1, Ca^{2+}-dependent and urea-resistant complex with calmodulin. The dissociation constant equals 1.6 nM. In the absence of Ca^{2+} a low-affinity complex is formed that is disrupted by 4 M-urea. On the basis of these properties, a fast affinity purification of seminalplasmin was developed. The high specificity of seminalplasmin as a calmodulin antagonist was demonstrated for the multipathway-regulated adenylate cyclase of bovine cerebellum. Far-u.v. c.d. properties are consistent with a random form of seminalplasmin in aqueous solution; 23% α-helix is induced on interaction with calmodulin. The fluorescence properties of the single tryptophan residue of seminalplasmin are markedly changed on formation of the complex. These studies allowed us to locate tentatively the peptide segment that interacts with calmodulin, and to ascertain the structural homology between seminalplasmin and other calmodulin-binding peptides. Additional material, showing the inhibition of calmodulin-mediated activation of bovine brain phosphodiesterase by melittin and seminalplasmin and also the near-u.v. spectrum of affinity-purified seminalplasmin, has been deposited as supplement SUP 50135 (4 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in Biochem. J. (1986) 233, 5.

INTRODUCTION

Seminalplasmin (SP) is an antimicrobial peptide [1] that coats the surface of ejaculated spermatozoa [2] and may promote the acrosome reaction [3], an obligatory event in fertilization. The peptide has been purified to electrophoretic homogeneity by classical chromatographic steps [1] or reverse-phase h.p.l.c. [4], and shown to be highly water-soluble and monomeric with a random distribution of hydrophobic and predominantly basic amino acid residues [4]. In this respect it resembles other basic amphiphilic peptides such as melittin and mastoparans. Interestingly, Gietzen & Galla [5] recently reported that SP, like the bee-venom cytolytic toxins, antagonizes specifically and with high potency the function of calmodulin (CaM), the major Ca^{2+} vector in eukaryotic cells [6,7]. In the case of melittin [8], mastoparans [9] and their synthetic homologues [10] it has been demonstrated that the antagonism results from the formation of a 1:1 Ca^{2+}-dependent complex of high affinity (K_D approx. 10^{-9} M) between the peptide toxins and CaM. The latter interaction has recently been studied in much detail [11–13] with the premise that the same type of forces may be operational in the interaction of CaM with its target enzymes [14]. The direct interaction of SP with CaM has been studied much less intensively: Gietzen & Galla [5] indicated that the peptide interacts with Sepharose-conjugated CaM in the presence as well as the absence of Ca^{2+} but is eluted with high-ionic-strength buffers, thus pointing to the electrostatic nature of the interaction. Here we report on the stoichiometry and the affinity constant of the complex between SP and CaM, on its Ca^{2+}-dependency and on some structural aspects of the interaction. The data indicate that the interaction of SP with CaM bears strong resemblance to the one between CaM and melittin, mastoparans and synthetic model peptides.

EXPERIMENTAL

Bovine brain CaM was purified by the procedure of Gopalakrishna & Anderson [15], followed by hydroxyapatite chromatography. [^{3}H]Monoacetyl-CaM and Sepharose 4B-immobilized melittin (Melex) were prepared as previously described [10]. Sepharose 6B-immobilized CaM with a capacity of 3.7 mg of protein/ml of wet resin was prepared as described by Kohn & Wilcheck [16]. SP was isolated by Sephadex A-25, CM-Sephadex C-50 and Sephadex G-75 chromatography as previously described [1] followed by affinity chromatography on Sepharose 4B-conjugated CaM as described in Results section. Urea-containing solutions were freshly prepared. During the purification procedure, SP was detected in the fractions by monitoring complex-formation with CaM by polyacrylamide-disc-gel electrophoresis in non-denaturing conditions as described previously for the case of melittin [8; see also Fig. 2]. The buffers used for discontinuous electrophoresis were those of Davis [17]. The purity was checked by h.p.l.c. on a μBondapak C_{18} column (Waters Associates, Milford, MA, U.S.A.), the elution being monitored at 210 nm (Spectraphysics SP 8100 instrument).

Competitive phosphodiesterase assays as well as the

Abbreviations used: SP, seminalplasmin; CaM, calmodulin; peptide LK2, N^{α}-9-fluorenylmethoxycarbonyl-(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_2$; Melex, Sepharose 4B-conjugated melittin.

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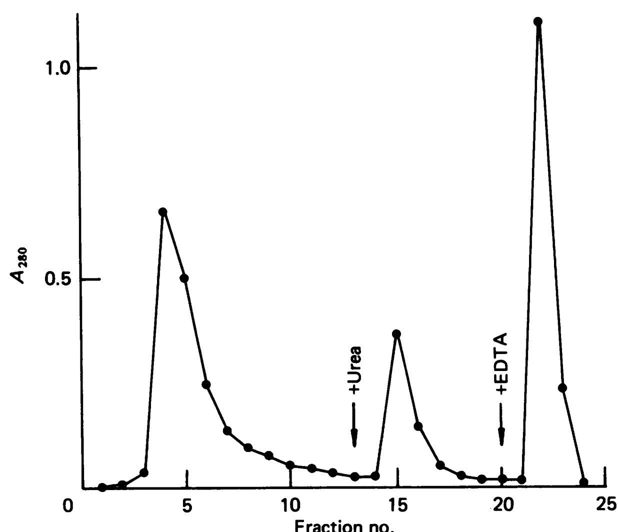

Fig. 1. Affinity chromatography of partially purified SP on Sepharose 4B-conjugated CaM (11 ml of resin)

The experimental conditions are outlined in the Results section. The absorbance was monitored at 280 nm. After elution of 13 fractions with the initial 50 mM-Tes/NaOH buffer, pH 7.0, containing 100 mM-NaCl and 1 mM-$CaCl_2$, elution was continued with the same buffer supplemented with 4 M-urea; after 20 fractions, all SP was quantitatively eluted with a 50 mM-Tes/NaOH buffer, pH 7.0, containing 100 mM-NaCl, 1 mM-EDTA and 4 M-urea.

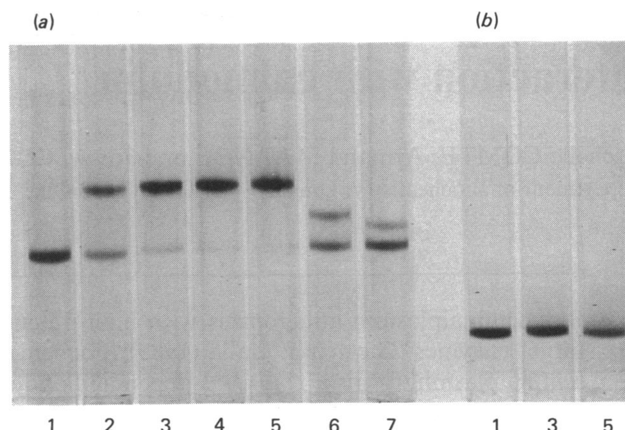

Fig. 2. Complex-formation between CaM and SP monitored by 12.5%-polyacrylamide-gel electrophoresis in the presence of 1 mM-$CaCl_2$ (*a*) or 1 mM-EDTA (*b*)

All samples contain 0.18 nmol of CaM. Lane 1, CaM; lane 2, +0.09 nmol of SP; lane 3, +0.18 nmol of SP; lane 4, +0.36 nmol of SP; lane 5, +0.54 nmol of SP; lane 6, +0.09 nmol of melittin; lane 7, +0.04 nmol of peptide LK2. Free SP, melittin and peptide LK2 do not migrate in this electrophoretic system.

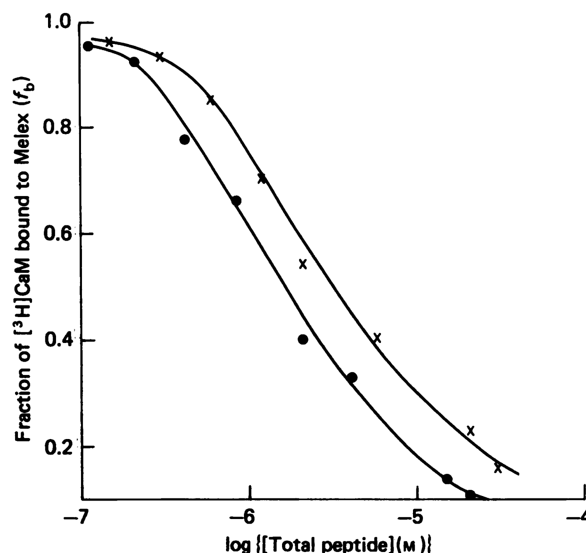

Fig. 3. Displacement of Melex-bound [^{3}H]CaM by SP as compared with melittin

The incubation mixture contained 3.9 μM immobilized melittin units (10 μl of settled Melex gel/250 μl assay volume), 170 nM-CaM, trace amounts of [^{3}H]monoacetyl-CaM and increasing amounts of soluble melittin (×) or SP (●). The assay buffer was composed of 60 mM-Tes/NaOH buffer, pH 7.0, containing 135 mM-NaCl and 1 mM-$CaCl_2$ and the amount of Ca^{2+}-sensitive complex between CaM and Melex (f_b) was determined as described previously [10].

competitive Melex assay were carried out as previously described [10]. The preparation of cerebellar membranes and adenylate cyclase assays were carried out as described by Malnoë & Cox [18]. Fluorescence and c.d. measurements were performed as previously described [10].

Protein and peptide concentrations were determined spectrophotometrically by using the following molar absorption coefficients: $\epsilon_{278} = 3020\ M^{-1}\cdot cm^{-1}$ for metal-ion-free CaM, $\epsilon_{280} = 5500\ M^{-1}\cdot cm^{-1}$ for melittin and 6970 $M^{-1}\cdot cm^{-1}$ for SP [19].

RESULTS

Purification of SP on CaM–Sepharose 4B

After the three classical chromatographic steps mentioned in the Experimental section SP still contained a number of contaminating proteins and/or peptides. On the basis of the finding by Gietzen & Galla [5], we devised a further purification procedure with the use of chromatography on immobilized CaM. A 20 mg portion of crude SP in 50 mM-Tes/NaOH buffer, pH 7.0, containing 100 mM-NaCl and 1 mM-$CaCl_2$ was loaded on a 10 ml column of immobilized CaM (3.7 mg of protein/ml bed volume) equilibrated in the same buffer. The column was washed successively with the same buffer without and with 4 M-urea without any release of SP; the latter was eluted quantitatively with 50 mM-Tes/NaOH buffer, pH 7.0, containing 100 mM-NaCl, 4 M-urea and 1 mM-EDTA (Fig. 1). The SP-containing fractions (fractions 21–23) were desalted immediately on Sephadex G-25 equilibrated in 5 mM-ammonium acetate buffer, pH 7.0, and freeze-dried. On h.p.l.c. (see the Experimental section) SP was eluted as a single peak with a purity above 99%.

Stoichiometry of the CaM-SP complex

Fig. 2 shows that CaM forms a complex with SP that is stable during electrophoresis and has a distinctly lower mobility than CaM alone. In this respect, SP is

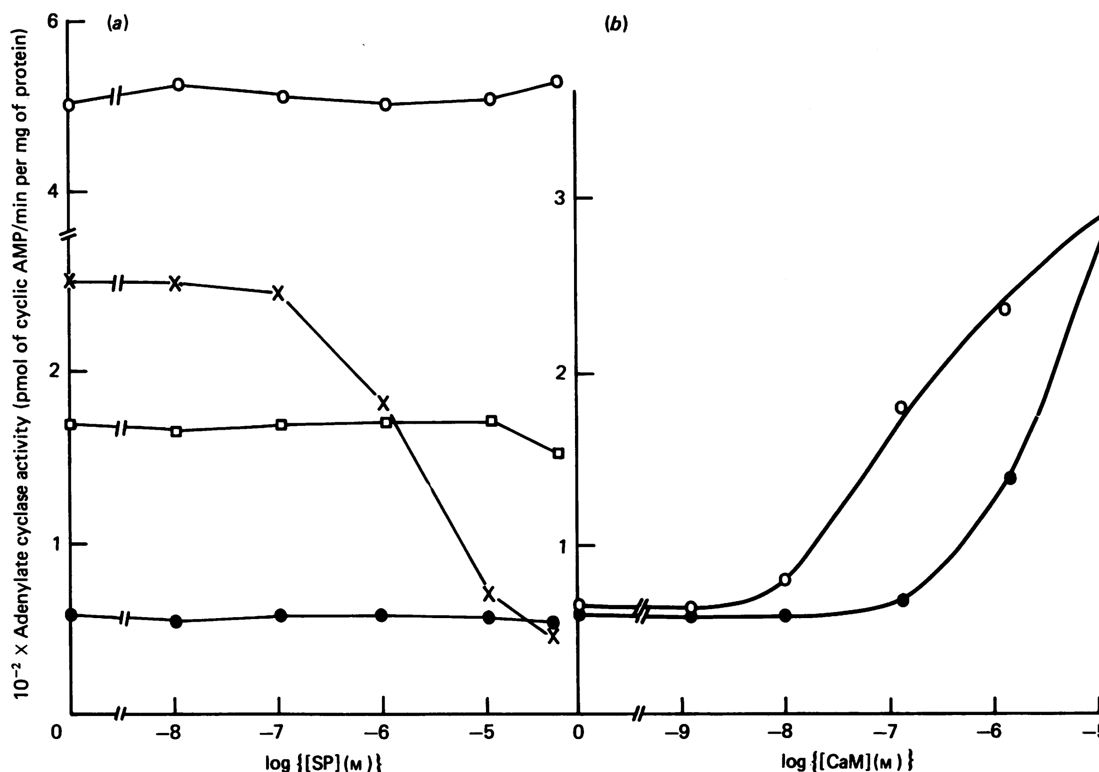

Fig. 4. Effect of SP on the adenylate cyclase activity in bovine cerebellar membranes

(*a*) The assay buffer contained 1 mM-EGTA. The enzyme assays were carried out at increasing concentrations of SP in the presence of the following effectors: ●, none; □, 50 μM-GTP + 50 μM-(−)isoprenaline; ×, 0.5 μM-CaM + 0.9 mM-$CaCl_2$; ○, 50 μM-forskolin. (*b*) Inhibition of CaM-mediated activation of adenylate cyclase by SP. The enzyme activity was measured as a function of the CaM concentration in the absence (○) or in the presence (●) of 5 μM-SP. The assay buffer contained 0.9 mM-$CaCl_2$ and 1 mM-EGTA.

similar to melittin [8] and different synthetic peptides [10,20], but not to δ-haemolysin, which forms a complex of lower affinity [10]. Interestingly, the complexes of CaM with SP, melittin and LK2 all have different mobilities on the gels, the mobility decreasing with increasing length of the peptide. At equimolar concentrations of SP and CaM nearly all CaM migrates as a complex. No band of lower mobility than that of this complex appeared at higher amounts of SP up to 3 equivalents/equivalent of CaM. These data indicate that CaM contains one single high-affinity site for SP. In the absence of Ca^{2+} (Fig. 2*b*) no such complex is formed, but at a high ratio of SP to CaM the intensity of the CaM band decreases without the appearance of a distinct new protein band on the gels. Similar behaviour has also been observed with melittin [8], where there is evidence of a non-specific and low-affinity association of one CaM molecule with four or more melittin molecules, especially in the low-ionic-strength buffer used for electrophoresis. Such a complex would either aggregate owing to the high pH (8.6) of the migration buffer, or even migrate in the opposite direction.

The 1:1 stoichiometry was confirmed by gel filtration on Sephadex G-75 equilibrated in 20 mM-Tes/NaOH buffer, pH 7.0, containing 100 mM-NaCl and 1 mM-$CaCl_2$ at 25 °C. For this purpose, a mixture of 60 nmol of [^{3}H]monoacetyl-CaM (65000 c.p.m./mg) and 90 nmol of SP was loaded on the column (60 cm × 0.9 cm) at a rate of 250 μl/min and the eluate was monitored for [^{3}H]CaM and for the absorbance at 280 nm (elution profile not shown). It should be noted that, as was documented previously in the case of the melittin–CaM [8] and peptide LK2–CaM complexes [10], the SP–CaM complex does not dissociate to a significant extent during gel-filtration chromatography even when the column buffer does not contain a background amount of SP. From these data it can be calculated that 55 nmol of SP was eluted in association with CaM in the void volume, whereas the remaining free SP was retarded. A stoichiometry of 0.92 equivalent of SP/equivalent of CaM was thus revealed.

Determination of the affinity of SP for CaM

Melex is a convenient matrix for the determination of the affinity of CaM for peptides [10,20]. Fig. 3 illustrates the displacement of CaM from the Melex resin by SP and by free melittin. These displacement curves are related to the dissociation constants by the following equation [10]:

$$K_{SP}/K_{ME} = \{[SP_T]-[CaM_T](1-f_b)\}/\{[ME_T]-[CaM_T](1-f_b)\} \quad (1)$$

where $[SP_T]$ and $[ME_T]$ are total concentrations of SP and melittin required for a given displacement $(1-f_b)$ of CaM from the resin, and $[CaM_T]$ is the total CaM concentration. From the known dissociation constant of 3 nM, in the presence as well as in the absence of 4 M-urea [8], for K_{ME}, a value of 1.6 nM was calculated for the dissociation constant of SP. A similar experiment in the presence of 4 M-urea (not shown) yielded a K_{SP} of 2.3 nM.

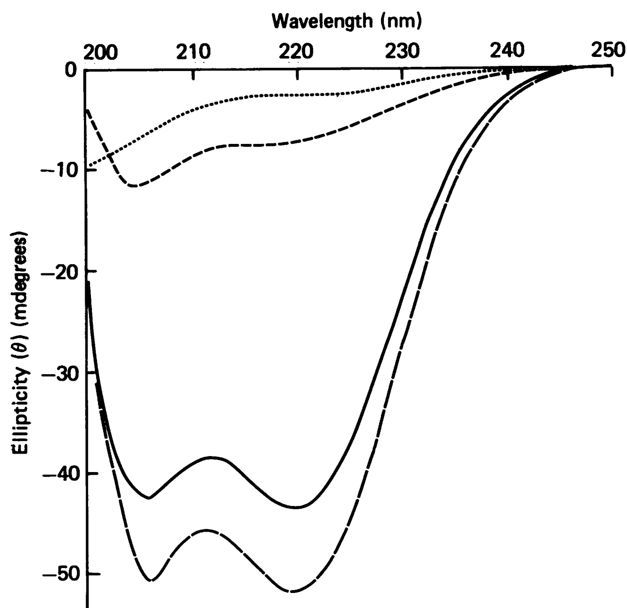

Fig. 5. Far-u.v. c.d. spectra of CaM and its complex with SP

All spectra were recorded on a Jasco J20 spectropolarimeter at room temperature with a slit width of 2 nm in a 0.2 cm-light-path quartz cuvette. The buffer was 60 mM-Tes/NaOH buffer, pH 7.0, containing 135 mM-NaCl and 1 mM-$CaCl_2$. ——, 10 μM-CaM; ——, 10 μM-CaM+10 μM-SP; ······, 10 μM-SP; ----, 10 μM-SP in buffer containing 0.3% SDS.

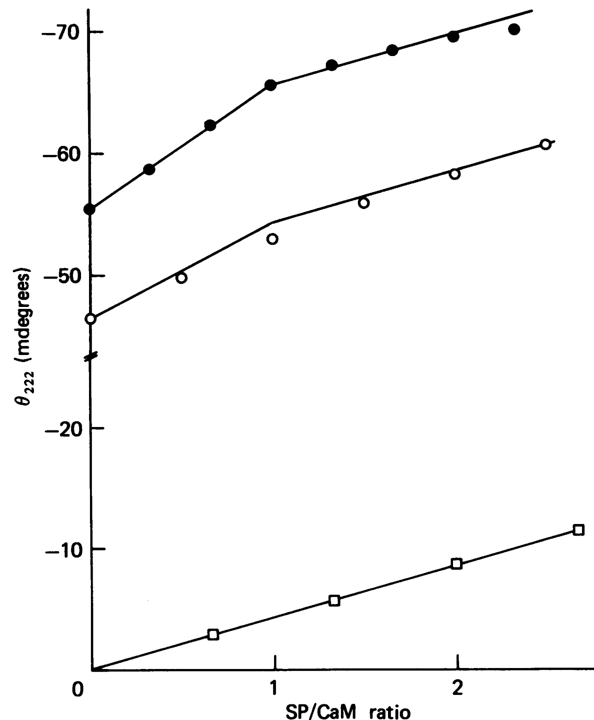

Fig. 6. Ellipticity titration of 10 μM-CaM by SP in the presence of 1 mM-$CaCl_2$ (●) or 1 mM-EGTA (○)

The experimental conditions were as indicated in Fig. 5 legend. In the control experiment with SP alone (□), the abscissa corresponds to a concentration of 10 μM for 1 unit. The continuous lines are computed stoichiometric titration curves for the binding of 1 molecule of SP/molecule of CaM, assuming no conformational change in CaM, 23% α-helix increase in bound SP in the presence of $CaCl_2$ and 12% in the presence of EGTA.

Enzyme inhibition by SP

The first evidence for a high-affinity complex-formation between CaM and SP was based on inhibition experiments on the CaM-dependent enzymes brain phosphodiesterase and erythrocyte $Ca^{2+}+Mg^{2+}$-dependent ATPase [5]. The fact that SP and melittin have essentially the same affinity for CaM (Fig. 3) has been confirmed in the phosphodiesterase assay with 100 nM of each peptide (Supplement SUP 50135). The specific effect of SP on CaM-activated processes was further demonstrated on cerebellar adenylate cyclase, an enzyme with a complex pattern of regulation by guanine nucleotides, CaM, forskolin and Ca^{2+} [18,21]. Fig. 4(*a*) shows that, up to 50 μM, SP is without any significant effect on the basal activity of adenylate cyclase, or on the stimulatory pathways via forskolin and the guanine nucleotide-binding component. Similarly, SP has no effect on Ca^{2+}-inhibited (1 mM free Ca^{2+}) adenylate cyclase (results not shown). However, the stimulatory effect of CaM (0.5 μM) in the presence of micromolar free Ca^{2+} is specifically and fully antagonized by SP (IC_{50} approx. 1 μM). Interestingly, the synergistic activation by CaM plus forskolin [18] also is abolished by SP and diminished to that of forskolin alone at 10 μM of the peptide (results not shown). The specificity of SP is further demonstrated by the fact that its inhibitory effect at 5 μM could be completely reversed by increasing concentrations of CaM up to 10 μM (Fig. 4*b*).

Structural characteristics of the interaction between SP and CaM

Far-u.v. c.d. has been used to show that various peptides become more α-helical on interaction with CaM [10–12,20–22]. Fig. 5 shows that SP is in a random-coil conformation with a mean residue ellipticity at 222 nm of −3000 degrees·cm²·dmol⁻¹. Ca^{2+}-saturated CaM has a $[\theta]_{m.r.w.}$ value of −14700 degree·cm²·dmol⁻¹ under similar conditions. When both the partners were mixed in equimolar amounts, the ellipticity minima at 222 and 208 nm increased more than the sum of each taken separately, indicating that complex-formation enhances its α-helical content. The difference spectrum between the complex and CaM is quantitatively similar to the spectrum of SP alone in 0.3% SDS (Fig. 5). In analogy with the case of melittin [11], this strongly suggests that the observed enhancement of negative ellipticity in the complex is essentially due to an induction of α-helix in SP, and not in CaM. If so, the mean residue ellipticity at 222 nm of bound SP is −6100 degree·cm²·dmol⁻¹, which corresponds to approx. 23% α-helix content [23]. Fig. 6 shows the ellipticity titration of 10 μM-CaM with SP in the presence and in the absence of Ca^{2+}. As expected, provided that Ca^{2+} is present, the ellipticity increase levels off at a ratio of 1:1. At a SP/CaM ratio above 2:1 the titration curve slightly bends downwards, perhaps owing to the denaturing effect of the amphiphilic peptide [11]. In the absence of Ca^{2+} the increase in ellipticity on titration of CaM with SP is also more than additive (Fig. 6), but the effect is less pronounced and the interaction displays a much lower affinity. The existence of a low-affinity complex between metal-ion-free CaM

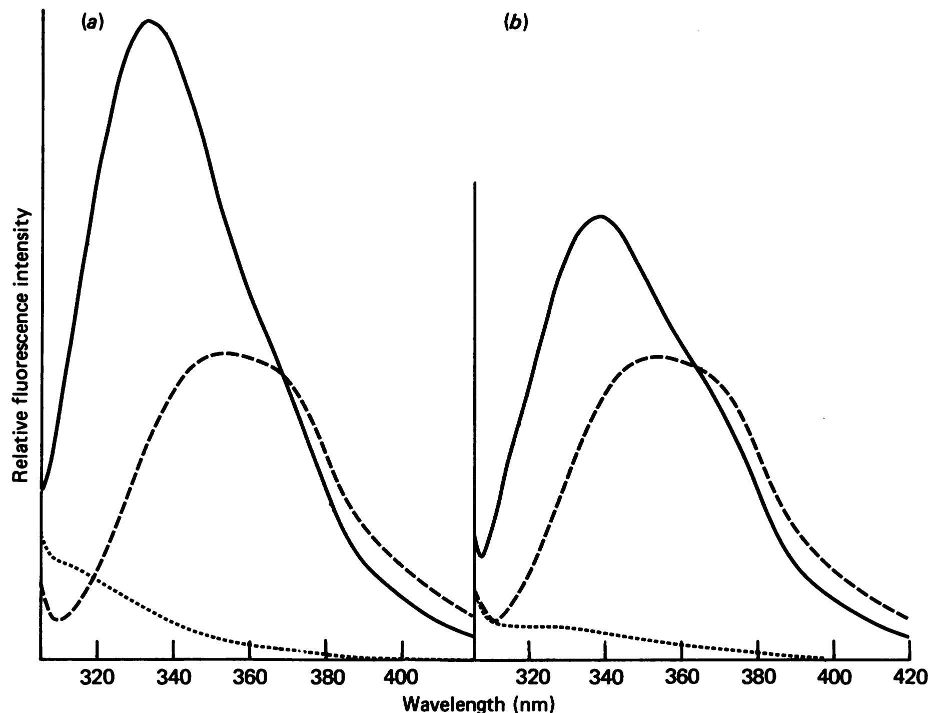

Fig. 7. Tryptophan emission fluorescence spectra of SP and CaM on complex-formation

The spectra were recorded on a Baird Nova spectrofluorimeter at 25 °C with the excitation wavelength at 290 nm. (*a*) Measurements in 60 mM-Tes/NaOH buffer, pH 7.0, containing 135 mM-NaCl and 1 mM-$CaCl_2$. ----, 30 μM-SP; ······, 30 μM-CaM; ——, 30 μM-SP+30 μM-CaM. (*b*) Same as (*a*) except that 1 mM-$CaCl_2$ was neutralized by 2 mM-EDTA.

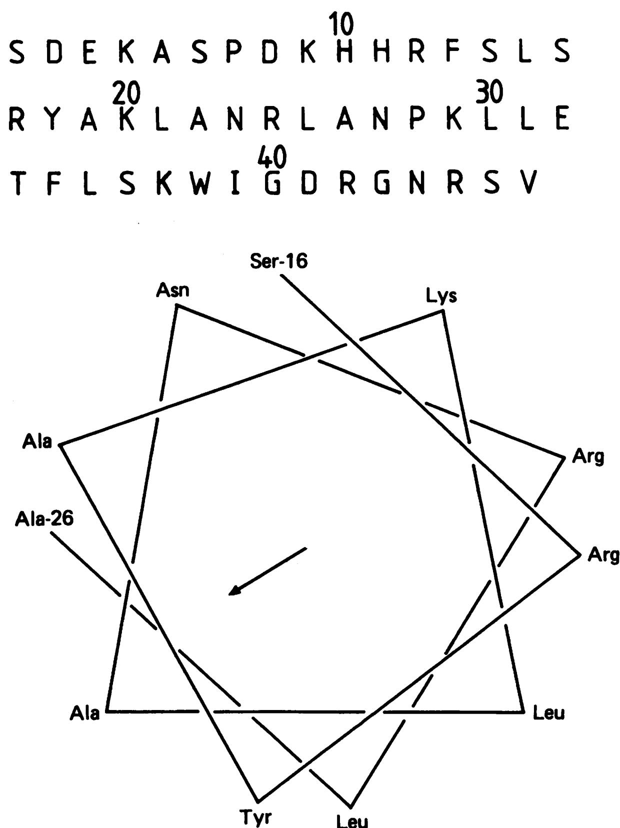

Fig. 8. Amino acid sequence of bull SP taken from [4] and helical wheel diagram for the segment Ser-16 to Ala-26

The arrow indicates the direction of the mean hydrophobic moment.

and SP has already been suggested by Gietzen & Galla [5], and was also observed in the case of melittin [11].

SP possesses one tryptophan residue in position 28. It was therefore of interest to measure whether this probe is affected by the interaction with CaM. Fig. 7 shows the fluorescence spectra of CaM, SP and their 1:1 complex in the presence of 1 mM-$CaCl_2$. Most remarkable is the blue-shift of the fluorescence maximum from 350 nm (corresponding to a tryptophan residue freely mobile in water) to 335 nm (corresponding to the immobilization of this tryptophan residue), as well as a 2.5-fold increase in fluorescence intensity. Blue-shifts of the tryptophan fluorescence on binding of model peptides to CaM have systematically been observed [11,20,24], but in these cases the fluorescence quantum yield was not noticeably changed. In contrast, δ-haemolysin [10] and SP [present study] display a more than 2-fold increase in fluorescence intensity. In this respect, it should be noted that there is some sequence homology around the tryptophan residue in both peptides. Fig. 7 also shows that the low-affinity complex, obtained on neutralization of Ca^{2+} by 2 mM-EDTA, displays a less pronounced blue-shift (from 350 to 339 nm) and a weaker enhancement of the tryptophan fluorescence (1.5 instead of 2.5).

DISCUSSION

Affinity chromatography on immobilized CaM is a very convenient way for the fast purification of over 99% pure SP, which displays a near-u.v. absorption spectrum typical for the presence of one tryptophan and one tyrosine residue (Supplementary Publication SUP 50135). The affinity-chromatography step removed essentially one endogenous protein component that must

Table 1. Amino acid sequences of (putative) CaM-binding domains in peptides or proteins with approx. 10^9 M^{-1} affinity (series A) or 5×10^7–10^8 M^{-1} affinity (series B) for CaM

Boxed residues are the invariant hydrophobic residues.

	1	2	3	4	5	6	7	8	9	10	11	12	13	Reference*
Series A														
Seminalplasmin	^{14}S	L	S	R	Y	A	K	L	A	N	R	L	A	[4,31]
Melittin	^{12}G	L	P	A	L	I	S	W	I	K	R	K	R	[14]
Mastoparan	^{2}N	L	K	A	L	A	A	L	A	K	K	I	L	[9]
Helodermin	^{6}F	T	Q	Q	Y	S	K	L	L	A	K	L	A	[27]
Peptide LK2		^{1}L	K	K	L	L	K	L	L	K	K	L	L	[10]
Peptide IV	^{2}K	W	K	K	L	L	K	L	L	K	K	L	L	[20]
Peptide M13	^{9}I	A	V	S	A	A	N	R	F	K	K	I	S	[26]
Peptide RS20	^{6}K	T	G	H	A	V	R	A	I	G	R	L	S	[24]
Series B														
P. Mastoparan	^{2}D	W	K	K	I	G	Q	H	I	L	S	V	L	[9]
δ-Haemolysin	^{8}T	I	G	D	L	V	K	W	I	I	D	T	V	[28]
Vasoactive peptide	^{11}Y	I	L	R	K	Q	M	A	V	K	K	Y	L	[29]
Troponin I	^{7}A	I	T	A	R	R	Q	H	L	K	S	V	M	
	^{103}R	G	K	F	K	R	P	P	L	R	R	V	R	[30]

* In each reference the whole amino acid sequence of the peptide or protein is represented.

interact weakly with SP, since after this chromatography SP is no longer retained on dialysis membranes with an 8000-M_r 'cut-off'. The latter contaminant may be identical with the 'anti-SP' described by Reddy & Bhargava [1]. Elution of SP from immobilized CaM requires the presence of both EDTA and 4 M-urea in the elution buffer. The requirement for urea stems from the observation that a non-specific low-affinity complex exists between CaM and SP in the absence of Ca^{2+} (Fig. 6); the latter, in contrast with the Ca^{2+}-sensitive complex, is disrupted by urea. This behaviour is reminiscent of the interaction of CaM with melittin [8,11].

In many aspects, the complex-formation between SP and CaM is identical with that of melittin, mastoparans and synthetic model peptides: the 1:1 Ca^{2+}-dependent complex is fully resistant to urea and displays an affinity constant of approx. 10^9 M^{-1}. Furthermore, like the other peptides, SP is in a fully random-coil conformation in the absence of CaM or other helix-promoting solvents, but the interaction with CaM leads to the induction of α-helix in the peptide. It should be noted that there are some qualitative differences in the α-helical induction in the different peptides; if entirely attributed to the peptide, the extent of α-helix induction and the number of amino acid residues involved are the following: for melittin, 72% increase, 19 residues involved [11]; for mastoparans, 51–61% increase, seven to nine residues involved [12]; for synthetic peptide II (previously called LK2), 50% increase, seven residues involved [20]; for the synthetic peptides IV and V, 65% increase, 11 residues involved [20], for SP, 23% increase, 11 residues involved [present study]. For comparison, the α-helix increase on interaction with CaM in the CaM-binding peptide RS20, originating from gizzard myosin light-chain kinase, involves 12 residues [24]. We [10] and others [12] have recently suggested that an amphiphilic α-helix of about three turns (11 residues) and containing several positively charged residues is favourable for high-affinity interaction with CaM. Therefore the amino acid sequence of SP (Fig. 8). was analysed with the hydrophobicity and hydrophobic moment $<\mu_H>$ plot according to the method of Eisenberg *et al.* [25] with the use of an 11-residue 'window'. The most characteristic segment is the one spanning Ser-16 to Ala-26 (Fig. 8). With its $<\mu_H>$ of 0.725 and a mean hydrophobicity $<H>$ of -0.298, this stretch must, like δ-haemolysin [24], melittin [24] and LK2 [24] and mastoparans [12], be labelled 'surface-seeking'. Moreover, this stretch contains three positive charges, scattered over the helix, and is thus likely to be involved in the interaction with CaM. Interestingly, this segment contains the single tyrosine residue of SP. Since in the fluorescence experiment (Fig. 7) predominantly the tyrosine residues are excited and as a result the tryptophan fluorescence properties are markedly changed, we assume that in the complex the Tyr–Trp distance is within the limits of efficient energy transfer. When the primary structures of the above-mentioned peptides, as well as of the peptides generated from natural CaM-target enzymes [24,26], are compared (Table 1), it is obvious that sequence homology is not required for high-affinity interaction with CaM; however,

their secondary and tertiary structures are invariably well conserved. Table 1 updates previous minimal-structural-requirements predictions about high-affinity CaM-binding domains [10]: very interesting are the invariant hydrophobic residues in positions 2, 5, 6, 9, 12 and 13 and an invariant positive charge in position 11; deviations from this theme lead to a decrease of the affinity, as shown in the B series of Table 1. A screening of the protein sequence data bank for this particular structure might uncover new unexpected targets of CaM.

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