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A comparison of the properties of the binary and ternary complexes formed by calmodulin and troponin C with two regulatory peptides of phosphorylase kinase

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Abstract

The regulatory peptides Phk13 (301–327) and a modified form of Phk5 (342–367) from the γ-subunit of glycogen phosphorylase kinase form binary and ternary complexes with both calmodulin and the related muscle protein troponin C. Neither peptide appears to affect to a major extent a fluorescent probe linked to Cys-27 of wheat germ calmodulin. Phk13, but not Phk5, significantly modifies the properties of a probe joined to Cys-98 of troponin C. A comparison by means of radiationless energy transfer of the average separations of Trp-16 of Phk5 from specific groups in the N- and C-terminal halves of calmodulin and troponin C indicate significant changes upon going from the 1:1 binary complex to the 1:1:1 ternary complex with Phk13. A comparison of the effects of addition of Phk13 to calmodulin, troponin C, and their binary complexes with Phk5 suggests that the conformation of Phk13 is similar in the binary and ternary complexes.

Keywords: Phosphorylase kinase; Calmodulin; Troponin C; Regulatory peptides; Binary complexes; Ternary complexes

1. Introduction

This paper is intended as a tribute to Bill Harrington, with whom the principal author (RFS) has had an association extending over 40 years. He has benefitted greatly, both personally and professionally, from this association. It is impossible to overstate the positive effects of interaction with Bill, whose resourceful mind was an endless source of ideas and suggestions.

The ubiquitous Ca^{2+} binding regulatory protein calmodulin combines with, and controls the activities of, a large variety of enzymes [1,2]. In several of the cases which have been studied in detail, the mechanism of regulation involves the interaction of calmodulin with an amphipathic regulatory peptide within the enzyme [3,4]. Upon combination with calmodulin the latter undergoes a major change in structure to assume an α-helical conformation within the complex [3]. The perhaps best characterized example is the interaction of calmodulin with the M13 regulatory peptide of the myosin light chain kinase of skeletal muscle, which causes the activation of the enzyme [5,6]. In complexes of this kind the calmod-

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ulin adopts a relatively compact shape [7–9]. The γ-subunit of glycogen phosphorylase kinase contains two non-contiguous peptides, both of which form complexes with calmodulin with high affinity [10]. However, the complexes have significantly different properties. The complex of calmodulin formed with Phk5 (342–367) has been reported from low-angle X-ray and neutron diffraction studies to involve an overall contraction of calmodulin to a relatively compact structure [11]. In contrast, Phk13 (301–327) has been reported to cause an extension, rather than a contraction [11]. The 1:1:1 ternary complex of calmodulin with both peptides has been found to have a spatial extension similar to that of the binary complex formed with Phk13 [11].

Subsequent work has confirmed that substantial differences exist between the two types of complex [12]. The complex formed with Phk5, but not that formed with Phk13, protects the connecting strand of calmodulin from proteolytic cleavage by trypsin at its midpoint, the 77–78 bond. Measurements of fluorescence anisotropy decay indicate a larger global correlation time for the Phk 13 complex, in harmony with expectations if the latter is spatially more extended and asymmetric [12].

In the case of the Phk13 complex, circular dichroism and radiationless energy transfer evidence suggest that the peptide has a low helical content within the complex and is moreover bent into a roughly hairpin-like conformation [12].

It is the purpose of the present communication to extend these studies to the ternary 1:1:1 complex with particular regard to a comparison of its properties with the binary species. A secondary purpose is to make comparative studies with the complexes formed with troponin C.

2. Experimental

2.1. Materials

Calmodulin. Bovine testes calmodulin was prepared by the method of Watterson et al. [13]. Wheat germ calmodulin was prepared by the method of Strasburg et al. [14]. Both preparations were homogeneous by the criterion of sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Chemical modification of calmodulin. Selective nitration of Tyr-99 of bovine calmodulin was carried out by the action of tetranitromethane in the absence of Ca^{2+}, according to the procedure of Richman and Klee [15]. Nitration of Tyr-139 of wheat germ calmodulin was carried out as described by Richman and Klee, after substitution of Cys-27 with iodoacetamide [15]. *N*-Acetylaminoethyl-5-naphthylamine-1-sulfonic acid (AEDANS) substitution of Cys-27 was carried out in 6 M guanidine hydrochloride, as described by Strasburg et al. [14].

The extent of labeling was determined spectrophotometrically, assuming values of $\varepsilon_{282} = 2200$ for nitrotyrosine and $\varepsilon_{337} = 6000$ for AEDANS. For this purpose calmodulin concentrations were determined by comparative measurements of ellipticity at 220 nm in the presence of Ca^{2+}, using native bovine calmodulin as a standard.

Troponin C. Troponin C (TnC) was prepared from frozen rabbit muscle by the procedure described in an earlier publication [16]. It was homogeneous by the criteria of SDS–acrylamide gel electrophoresis and acrylamide gel electrophoresis in 4 M urea. The AED–Cys-98 derivative of TnC was prepared by the reaction of IAEDANS with TnC by standard methods [17]. IAEDANS was purchased from Molecular Probes.

Peptides. All peptides used in this study were synthesized in the Department of Microbiology, School of Medicine, University of Maryland at Baltimore by the group headed by Dr. William Myers. An Applied Biosystems synthesizer was used, which employs 9-fluorenylmethoxycarbonyl as the protective reagent for α-amino groups.

The peptides were purified to homogeneity by reverse phase high-performance liquid chromatography (HPLC) using a Perkin-Elmer Cetus Instruments HPLC apparatus with a Waters C-18 column, employing 0.1% trifluoroacetic acid (TFA)–0.1% TFA–acetonitrile gradients. The homogeneity of the purified peptides was affirmed by HPLC patterns, amino acid sequencing, and mass spectra.

The Phk13 used in this study was modified in that Cys-8 was replaced by serine. In some experiments Phk13w4 was used, for which Phe-4 was replaced by tryptophan [12] (Table 1). A modified form of Phk5 was also used, supplied through the courtesy of Dr. John Collins (Table 1).

Table 1
Sequences of peptides used in this study

Peptide	Sequence
Phk13 (301–327) [a]	RGKF$_4$KVISLTVLASVRIYYQYRRVKPG
Phk13w4	---W$_4$---C---
Phk5 (342–363) [b]	C-KRLIDAYAFRIYGHWVRKGQQ

[a] The native sequence has been modified by the replacement of Cys-8 by Ser-8.
[b] The native sequence has been modified by the replacement of Leu-1 by Cys-1, Arg-2 by Lys-2, and Lys-18 by Arg-18.

2.2. Experimental conditions

Unless otherwise specified, all measurements reported here were performed in 0.05 M 3-(*N*-morpholine)propane sulfonic acid (Mops), 1 mM Ca^{2+}, 1 mM dithiothreitol, pH 6.5. The dithiothreitol was added to inhibit any cysteine oxidation. Concentrations of bovine testes calmodulin were determined from the ultraviolet absorption spectra, assuming a molar extinction coefficient at 276 nm of 3006 M^{-1} in the presence of Ca^{2+} and 3340 M^{-1} in its absence. Concentrations of wheat germ calmodulin were determined assuming a molar extinction coefficient at 280 nm of 1700 M^{-1}. Concentrations of troponin C were determined using an extinction coefficient at 276 nm of 4140 M^{-1}. Concentrations of the peptides were computed using molar extinction coefficients at 280 nm equal to the summed extinction coefficients of the Trp, Tyr, NO_2-Tyr, and Phe groups present in the peptide.

2.3. Methods

Static fluorescence. Fluorescence excitation and emission spectra were obtained using an SLM 48 000 spectrofluorometer. Emission spectra were corrected for the wavelength dependence of the photomultiplier tube.

Dynamic fluorescence. Time-domain dynamic fluorescence measurements using single photon counting were made using the instrument located in the Center for Fluorescence Spectroscopy at the University of Maryland at Baltimore (J.R. Lakowicz, Director). The light source was a mode-locked Nd–Yag laser driving a rhodamine laser, whose output was frequency-doubled. The time-domain measurements were analyzed by least-squares fits using the software supplied by the Edinburgh Company (Edinburgh, UK).

Intensity decay measurements were fitted to a function of the form

$$i(t) = \sum \alpha_i e^{-t/\tau_i} \quad (1)$$

Here, $i(t)$ is the fluorescence intensity as a function of time, t; α_i and τ_i are the amplitude and decay time, respectively, of the ith decay mode.

Circular dichroism. Circular dichroism spectra were measured using a Jasco 710 apparatus and a 1 mm path length cell. The optical system and cell compartment were continuously flushed with nitrogen gas. Measurements were routinely extended to 200 nm. The observed ellipticity values were converted to molecular ellipticities at wavelength λ, $[\theta_\lambda]$, by the equation

$$[\theta_\lambda] = 1000\,\theta_\lambda / cn_{aa} \quad (2)$$

where θ_λ is the observed ellipticity at wavelength λ, c is the molar concentration, and n_{aa} is the number of amino acids in the protein or peptide.

Radiationless energy transfer: static measurements. The efficiency, E, of radiationless energy transfer between a fluorescent donor group and an absorbing acceptor group is related to R, the separation of donor and acceptor groups, and R_0, the separation for 50% transfer efficiency by [18–20]

$$E^{-1} - 1 = \left(\frac{R}{R_0}\right)^6 \quad (3)$$

The quantity R_0 depends exclusively upon the properties of donor and acceptor groups and is given by [18]:

$$R_0^6 = \left[\{9000(\ln 10)\,K^2\Phi_D / 128\pi^5 N_0 n^4\} \int_0^\infty F_D(\lambda)\,\varepsilon_\lambda \lambda^4 \,d\lambda\right] \quad (4)$$

where Φ_D is the quantum yield of the donor in the absence of acceptor, N_0 is Avogadro's number, $F_D(\lambda)$ and ε_λ are, respectively, the normalized fluorescence intensity of the donor and the molar extinction coefficient of the acceptor at wavelength λ. The only quantity not accessible to direct measurement is K^2, the orientation factor, which, for random mutual

orientation of the donor and acceptor dipoles is equal to 2/3; this value is often assumed in the absence of direct information.

The quantum yield, Φ_D, of the donor was determined using free tryptophan as a standard, assuming a value of 0.14 for the quantum yield of the latter. The overlap integral $\int_0^\infty F_D(\lambda)\varepsilon_\lambda \lambda^4 d\lambda$ was determined by trapezoidal integration of $F_D(\lambda)\varepsilon_\lambda\lambda^4$ as a function of λ. The transfer efficiency E is equal to $1 - Y_{DA}/Y_D$, where Y_{DA} and Y_D are the values of the ratio $i_{333}/A_{295,D}$ for donor in the presence and absence of acceptor, respectively; i_{333} is the fluorescence intensity of the donor at 333 nm when excited at 295 nm and $A_{295,D}$ is the absorbance of the donor at 295 nm.

Transfer efficiency was also computed from dynamic fluorescence measurements of the time decay of fluorescence intensity. The efficiency of transfer is in this case given by $1 - \bar{\tau}_{DA}/\bar{\tau}_D$, where $\bar{\tau}_{DA}$ and $\bar{\tau}_D$ are equal to $\Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i$ for donor in the presence and absence of acceptor, respectively. Transfer efficiencies and separations computed in this way were in reasonable agreement with those computed from static measurements. In one case transfer efficiencies were also computed from $1 - \langle \tau_{DA} \rangle / \langle \tau_D \rangle$, where $\langle \tau \rangle = \Sigma \alpha_i \tau_i^2 / \Sigma \alpha_i \tau_i$.

Distribution of separations. If, instead of a constant separation of donor and acceptor groups, there is a distribution of separations characterized by a probability distribution function P_r, then the time decay of donor intensity in the presence of acceptor, i_{DA}, is given by [21–23]:

$$i_{DA}(t) = \int_0^\infty \sum_i \alpha_i \exp\left\{-(t/\tau_i) \times \left[1 + (R_0/R)^6\right]\right\} P_r dR \qquad (5)$$

The form of P_r is not known à priori. Both Gaussian and Lorentzian functions have been assumed in particular cases. It will here be assumed to have a Gaussian form [22]

$$P_r = \frac{1}{\sigma'\sqrt{\pi}} \left\{ \exp \frac{(R - \bar{R})^2}{\sqrt{2}\,\sigma'} \right\} \qquad (6)$$

where σ' is the standard deviation and $\bar{R}$ is the average separation.

In terms of the reduced quantities $\sigma (= \sigma'/R_0)$, $u (= R/R_0)$, and $\bar{u} (= \bar{R}/R_0)$, and making the substitution $z = (u - \bar{u})/\sqrt{2}\,\sigma$,

$$i_{DA}(t) = \frac{1}{\sqrt{\pi}} \int_{z^*}^\infty \sum_i \alpha_i \exp\left\{ -\left(\frac{t}{\tau}\right) \times \left[1 + \left(\bar{u} + \sqrt{2}\,\sigma z\right)^{-6}\right]\right\} e^{-z^2} dz \qquad (7)$$

where $z^* = -\bar{u}/\sqrt{2}\,\sigma$. Values of $\bar{u}$ and σ are identified by least-squares fitting of the time decay of i_{DA}.

Gel exclusion chromatography. HPLC measurements utilizing gel exclusion were made with a Perkin-Elmer Cetus HPLC apparatus and an Omnifit column filled with Sephacryl-100 (Pharmacia). The equilibration of the column with buffer and the technique of measurement followed closely the procedure of Nenortas and Beckett [24].

3. Results

Tryptophan fluorescence. The addition of a 1:1 calmodulin–Phk13 mixture to Phk5 results in a progressive shift of the emission maximum from 350 nm to 333 nm (Fig. 1). The change is complete at a 1:1:1 mole ratio of the three species. Thus the incorporation of Phk5 into a 1:1:1 ternary complex results in a change of emission properties characteristic of a transition of tryptophan to a more non-polar, solvent-shielded microenvironment.

However, the combination of Phk5 with the binary complex does not obey a strictly 1:1 stoichiometry. If the interaction is monitored by the fluorescence intensity at 333 nm, the variation is non-linear at mole ratios of the binary complex to Phk5 less than 1.0, showing a pronounced convex curvature (Fig. 1). This is consistent with, and suggests that, the binary complex of Phk13 and calmodulin can combine with more than one molecule of Phk5. This is not surprising, since it has already been shown that calmodulin can bind more than one molecule of both Phk5 and Phk13 [12].

The above conclusion is confirmed by parallel experiments in which CaMbt is replaced by NO_2-Tyr-99-CaMbt, which quenches the tryptophan fluo-

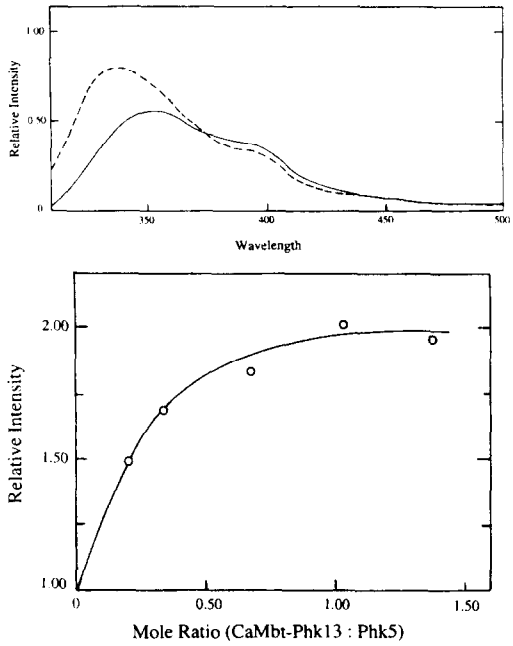

Fig. 1. (Top) Fluorescence emission spectra of Phk5 (6.0 μM) in the absence (—) and presence (---) of the 1:1 binary complex CaMbt–Phk13 (6.1 μM). The solvent is 0.05 M Mops, 1 mM DTT, 1 mM Ca^{2+}, pH 6.5. The excitation wavelength is 295 nm. (Bottom) Fluorescence intensity at 333 nm of Phk5 (6.9 μM) in the presence of varying levels of the 1:1 binary complex CaMbt–Phk13. Other conditions are the same as in Fig. 1 (Top).

rescence of Phk5. The variation of intensity with mole ratio of binary complex to Phk5 is non-linear, the degree of quenching approaching its limiting value at mole ratios of 0.5 (Fig. 2). For both native and nitro-calmodulin, the intensity is essentially constant at mole ratios greater than 1.0.

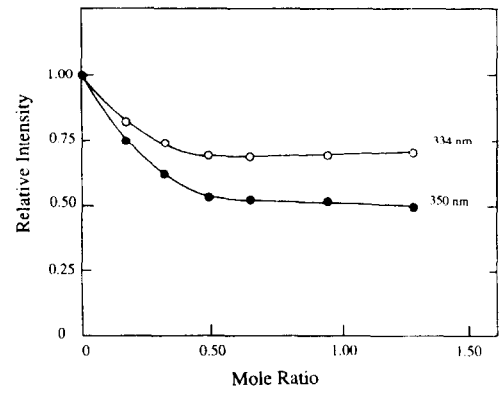

Fig. 2. Fluorescence intensity at 334 and 350 nm of Phk5 (6.6 μM) in the presence of varying levels of the 1:1 binary complex NO_2-Tyr-139-CaMwg–Phk13. Other conditions are the same as in Fig. 1.

The behavior observed here parallels that reported earlier for the interaction of Phk5 and Phk13 separately with calmodulin [12]. As in the latter case, the most plausible explanation is in terms of a single strong binding site for Phk5 within the binary complex, plus one or more weaker sites. If the value of K_d for the weaker site is in the range 1–10 μM, it could readily be detected in the concentration range of the present study.

A conceivable alternative explanation for the titration data and, in particular, for the curvature observed, might be in terms of the competitive displacement of Phk13 by Phk5. This would be qualitatively consistent with the convex curvature observed in the presence of excess Phk5. However, we believe that this model is unlikely for the following reasons.

Table 2
Transfer efficiencies and separations for binary and ternary complexes of CaM containing Phk5 [a]

Donor	Acceptor	Complex	R_0 (Å)	E_s [b]	R_s [b] (Å)	E_d [c]	R_d [c] (Å)	E_d · [d]	R_d · [d]
Trp-16	NO_2-Tyr-139	binary	18.4	0.61	17.1	0.43	19.3	0.29	21.3
		ternary	17.9	0.49	18.1	0.37	19.6	0.17	23.5
	AED–Cys-27	binary	17.8	0.32	20.3				
		ternary	17.4	0.28	20.4				

[a] The solvent is 0.05 M Mops, 1 mM DTT, 1 mM Ca^{2+}, pH 6.5.
[b] Transfer efficiencies and separations computed from static fluorescence measurements.
[c] Transfer efficiencies and separations computed from dynamic fluorescence measurements, using $\bar{\tau}$.
[d] Transfer efficiencies and separations computed from dynamic fluorescence measurements, using $\langle\tau\rangle$.

The reported binding constant of Phk13 is greater by a factor of 3 than that of Phk5 [10]. If Phk5 were competitively displacing Phk13 it would not be expected that the titration would be complete at a 1:1 mole ratio of Phk5 to the binary complex, as is observed (Fig. 1 and 2). Moreover, low-angle X-ray

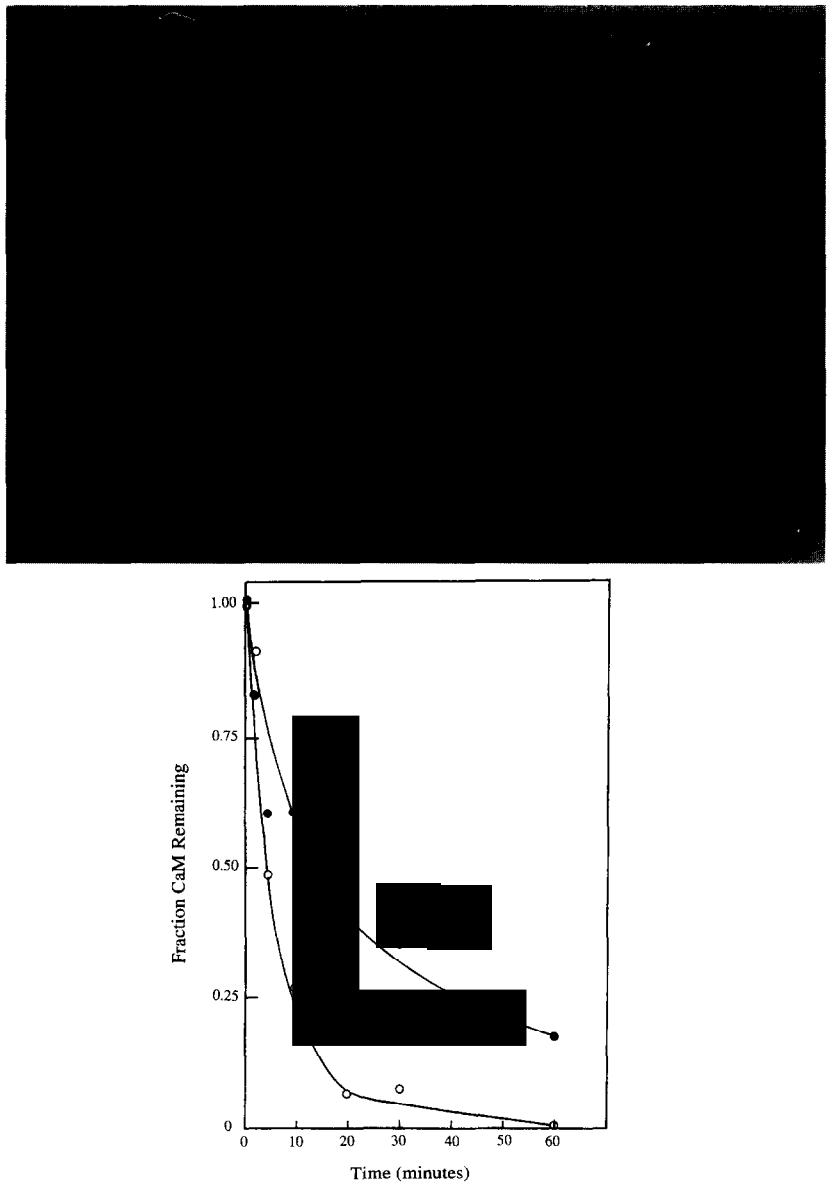

Fig. 3. (Top) Proteolysis by trypsin of CaMbt and the 1:1:1 ternary complex CaMbt–Phk13–Phk5. The concentrations of CaMbt and trypsin were 7.9 μM and 0.4 μM, respectively, in 0.1 M Hepes, 1 mM DTT, 1 mM Ca^{2+}, pH 7.5. The reaction was stopped by the addition of an equivalent of soy bean trypsin inhibitor to an aliquot of the reaction mixture. Acrylamide gel electrophoresis in 4 M urea was done for aliquots removed at increasing times. Lanes 1–7 (left) CaMbt alone: 0 m, 2 m, 5 m, 10 m, 20 m, 30 m, 60 m. Lanes 8–14 (right) 1:1:1 ternary mixture: 0 m, 2 m, 5 m, 10 m, 20 m, 30 m, 60 m. (Bottom) Relative intensities of CaMbt band as a function of time: (○) CaMbt alone; (●) CaMbt in 1:1:1 ternary mixture.

scattering indicates that the ternary complex is quite different in spatial geometry from the 1:1 binary complex with Phk5, resembling rather the 1:1 complex with Phk13 [11]. Finally, if Phk5 could displace Phk13, then Phk13 should displace Phk5 from a binary complex with CaM. If the latter were labeled with a group, such as NO_2-Tyr or AEDANS, which quenches Trp by radiationless energy transfer, then the addition of Phk13 in a 1:1 ratio to the binary complex of Phk5 with the CaM conjugate should result in the release of free Phk5 and a substantial increase in quantum yield and average decay time. This does not occur (Tables 2 and 4 and unshown data), even at a 3-fold excess of Phk13 (data not shown). Moreover, the addition of Phk13 to the Phk5–CaM binary complex in a 1:1 mole ratio does not cause a drop in fluorescence anisotropy, as would be expected if combined Phk5 were displaced and converted to the free peptide, but rather a slight rise in anisotropy (data not shown).

Tryptic digestion. In an earlier study it has been shown that formation of the 1:1 complex by Phk13 and calmodulin does not significantly slow the proteolysis of the latter by trypsin, while the 1:1 complex with Phk5 is substantially protected from proteolysis [12]. The behavior of Phk13 is in contrast to that of such amphipathic peptides as melittin, which protect against proteolysis [25].

Fig. 3 compares the proteolysis by trypsin of uncomplexed calmodulin and the 1:1:1 ternary complex. The latter is attacked at a significantly slower rate, indicating that the addition of Phk5 to the binary complex results in substantial protection of the 77–78 bond. This in turn suggests that Phk5 may be bound in proximity to the midsection of calmodulin, so as to shield the 77–78 bond. Alternatively, the protection may arise indirectly from an induced conformational change. However, the effect is much less pronounced than in the case of melittin [25].

State of association. The elution volume of CaMbt on Sephacryl-100 fell at approximately the expected position on a calibration curve generated by a series of standard proteins (data not shown). The elution volumes of the 1:1 binary complex with Phk13 and the 1:1:1 ternary complex with Phk13 and Phk5 did not differ from that of CaMbt by more than experimental uncertainty (data not shown). Moreover, acrylamide gel electrophoresis of both the binary and ternary complexes showed only single bands. The evidence for any major degree of self-association is thus negative.

Circular dichroism. The circular dichroism (CD) spectrum of combined Phk13 in the binary complex may be estimated by subtracting the spectrum of uncombined calmodulin from that of the complex and assuming that the spectrum of calmodulin itself

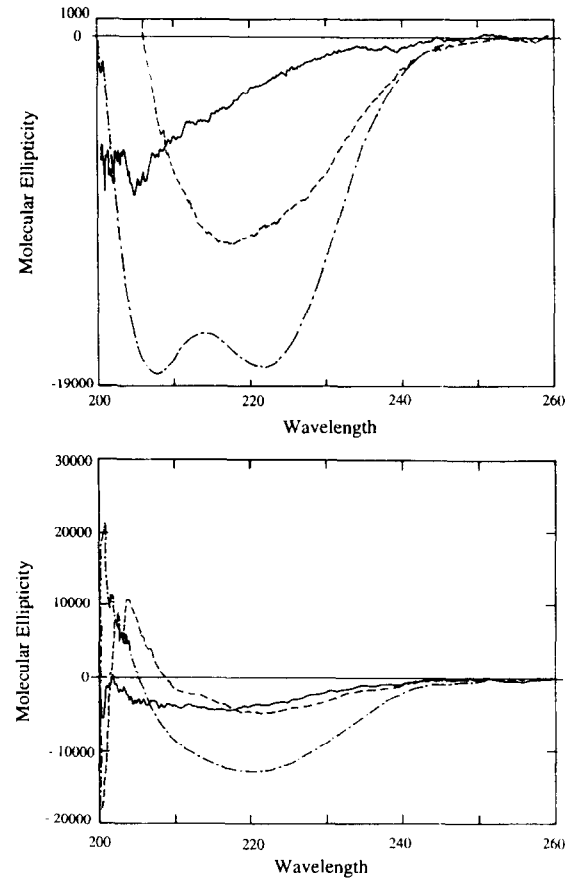

Fig. 4. (Top) Circular dichroism spectrum of Phk13 in the uncombined state (—); CaMbt (– · –); and the difference spectrum resulting from the subtraction of the CaMbt spectrum from that of the 1:1 complex of CaMbt and Phk13 (– – –). Molecular ellipticities are shown (degree cm^2 $decimole^{-1}$). (Bottom) Circular dichroism spectrum of Phk5 in the uncombined state (—); the difference spectrum resulting from the subtraction of the CaMbt spectrum from that of the 1:1 complex with Phk5 (– – –); and the difference spectrum resulting from the subtraction of the spectrum of CaMbt from that of the 1:1:1 complex of CaMbt, Phk5, and Phk13 (– · –). The solvent is 0.05 M Mops, 1 mM Ca^{2+}, 1 mM DTT, pH 6.5. All data are converted to molecular ellipticities.

is unchanged. The resulting difference spectrum is not characteristic of an α-helix, in contrast to the cases of such amphipathic peptides as M13 and melittin (Fig. 4). Qualitatively, it is more typical of a β-structure, showing only a single broad minimum near 215 nm, rather than two minima at 222 and 207 nm. There is, however, a substantial change from the spectrum of free Phk13 (Fig. 4), indicating a major change in structure upon combination with calmodulin.

It is worthy of mention that the CD spectrum of free Phk13 is significantly different from that of Phk13w4 [12]. The presence of a tryptophan at position 4 appears to modify the secondary structure of the free peptide.

If the spectrum of calmodulin is subtracted from that of the 1:1:1 ternary complex, the resultant difference spectrum, which represents the summed contributions of Phk13 and Phk5 within the ternary complex is likewise of a non-α-helical nature (Fig. 4).

Radiationless energy transfer. The donor group for radiationless energy transfer is Trp-16 of Phk5. The acceptor groups are AED–Cys 27 and NO_2-Tyr-139 of wheat germ calmodulin. For the binary complex of Phk5 and calmodulin the efficiencies of transfer are comparable for both acceptor groups (Table 2), although the efficiency is quantitatively greater for NO_2-Tyr-139. This is consistent with a location of Phk5 between the N- and C-terminal lobes of calmodulin such that Trp-16 is closer to Tyr-139 than to Cys-27. The efficiencies of transfer to NO_2-Tyr-139 are similar as computed from the quenching of donor static fluorescence intensity and from the decrease in the average decay time of donor fluorescence (Tables 2 and 3; Fig. 5).

The addition of Phk13 to form a 1:1:1 ternary complex results in a significant decrease in transfer efficiency to NO_2-Tyr-139, suggesting an increase in the separation of Trp-16 and NO_2-Tyr-139 (Table 2). In the case of the AED–Cys-27 derivative of wheat germ calmodulin (CaMwg), there is no change in separation from Trp-16 which is outside of experimental error (Table 2).

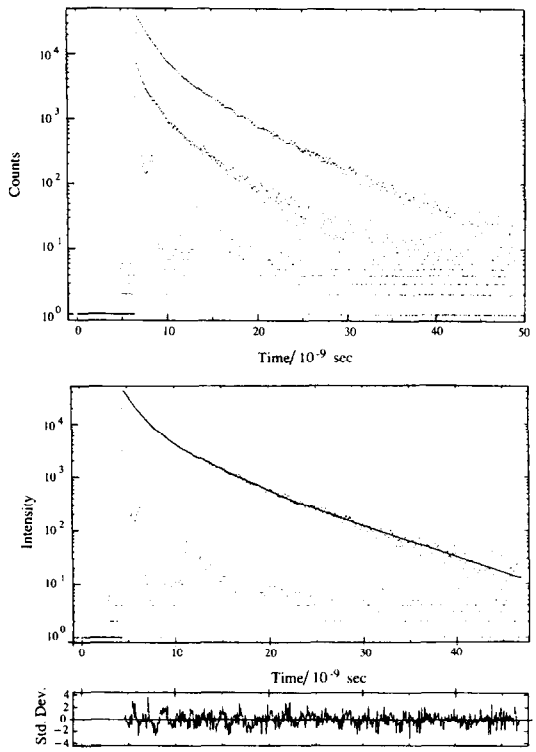

Fig. 5. (Top) A comparison of the time decay of fluorescence intensity for the 1:1:1 ternary complex of Phk5–Phk13 with CaMwg (upper curve) and NO_2-Tyr-139-CaMwg (lower curve). The concentration of each species is 13 μM. The excitation wavelength is 297 nm. A 335 nm cut-off filter intercepted the fluorescent beam. Other conditions are the same as in Fig. 1. (Bottom) A comparison of observed and computed data for the 1:1:1 ternary complex of Phk5–Phk13 with NO_2-Tyr-139-CaMwg. Conditions are the same as for Fig. 5 (top). The computed curve corresponds to the parameters cited in Table 3.

Table 3
Decay times of Trp-16 of Phk5 in absence and presence of acceptor groups [a]

Components present in complex	α_1	τ_1	α_2	τ_2	α_3	τ_3	$\bar{\tau}$	$\langle\tau\rangle$	χ^2
CaMwG	0.28	0.87	0.19	2.54	0.04	7.33	1.98	3.46	1.4
NO_2-Tyr-139-CaMwG	0.15	0.43	0.07	1.93	0.01	5.90	1.13	2.57	1.1
CaMwG + Phk13	0.35	0.97	0.20	2.65	0.04	7.52	1.96	3.33	1.2
NO_2-Tyr-139-CaMwG + Phk13	0.07	0.41	0.03	2.12	0.007	5.85	1.24	2.78	0.9

[a] The solvent is 0.05 M Mops, 1 mM DTT, 1 mM Ca^{2+}, pH 6.5.

A possible complication in the interpretation of the energy transfer data might be the presence of multiple bound molecules of Phk5 within the same complex, so that the observed emission represents a summation. While this model is difficult to disprove with finality, we feel it is unlikely for the following reasons. In the presence of an acceptor group linked to CaM, no further change occurs, outside of experimental uncertainty, in the relative intensity of Trp fluorescence as the ratio of the CaM–Phk13 binary complex to Phk5 is increased beyond 1:1 (Fig. 2 and unshown data). For the case of the NO_2-139-CaMwg derivative this was true for ratios in excess of 2:1 (data not shown). Thus, either any multiple bound molecules of Phk5 have equivalent transfer efficiencies, which is unlikely, or one binding site is much stronger than any others, so that at CaM–Phk13 to Phk5 mole ratios of 1.0 or greater, binding occurs preferentially to this site to the exclusion of the others. The latter explanation seems much more plausible and is consistent with findings for the binary systems [10–12]. Also, gel electrophoresis of the 1:1:1 complex indicated the presence of only one species rather than two or more, as would be expected if multiple binding were significant.

If the apparent distribution of separations between Trp-16 and NO_2-Tyr-139 in the binary and ternary complexes is computed using Eq. 7, it is found that the average separation is greater for the ternary species (Table 4). The standard deviation is quite large in both cases, increasing for the ternary complex. However, the fits were rather poor in the latter case, raising the possibility that the model is inadequate.

The significant differences in R, as computed by static and dynamic fluorescence measurements, is not surprising in view of the probable existence of a distribution of separations [27]. The transfer efficiencies computed from quantum yields and average decay times lead to average values of R. The kind of average is not equivalent for static and dynamic data; however, the difference between the binary and ternary complexes persists for both types of measurement. It is also of interest that the value of R, as determined with the use of Eq. 7, also increases for the ternary complex.

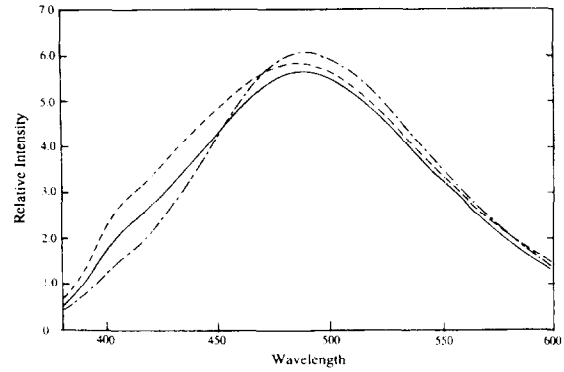

Fig. 6. Emission spectra of AED–Cys-27-CaMw6 (1.4 μM) in the absence of peptide (—), in the 1:1 complex with Phk13 (– · –), and in the 1:1:1 complex with Phk13 and Phk5 (– – –). The excitation wavelength is 340 nm. Other conditions are the same as in Fig. 1.

Microenvironment of AED–Cys-27. The addition of Phk13 to AED–Cys-27-CaMwg in a 1:1 ratio results in only a minor (ca. 5%) increase in intensity at λ_{max}, with no significant shift in the maximum wavelength. The further addition of Phk5 in a 1:1:1 ratio likewise produced only a slight (ca. 5%) intensity decrease with no maximum shift (Fig. 6).

Similarly, there was no major change in the accessibility of AED–Cys-27, as monitored by acrylamide quenching. The Stern–Volmer constant for quenching by acrylamide fell from 6.75 for the derivative in the absence of peptide to 4.9 for the 1:1 binary complex with Phk13 and to 4.4 for the 1:1:1

Table 4
Average separation and distribution of separations between Trp-16 of Phk5 and NO_2-Tyr-139

Components present in complex	$\bar{u}$	$\bar{R}$	σ	χ^2
NO_2-Tyr-139-CaMwg	0.97	17.8	0.59	2.94
NO_2-Tyr-139-CaMwg + Phk13	1.20	21.5	1.15	4.54

ternary complex with Phk13 and Phk5 (data not shown).

These results do not suggest a major involvement of either peptide in the microenvironment of AED–Cys-27. The modest changes observed could easily be accounted for in terms of the indirect local effects of an induced general conformational change. In particular, there is no significant further change on going from the binary to the ternary complex.

Interaction with troponin C. The Ca^{2+} binding muscle protein troponin C is similar in structure and physical properties to calmodulin and has been shown to combine in a similar manner with the basic amphiphilic peptide melittin [26]. By placing an AEDANS group on Cys-98 of TnC it is possible to monitor the microenvironment in the region near the junction of the connecting strand and the C-terminal lobe. Formation of a binary complex with TnC causes changes in the tryptophan emission spectra of Phk13w4 and Phk5 very similar to those produced by CaM (data not shown) [12].

Formation of the 1:1 complex of Phk13 with AED–Cys-98-TnC results in a substantial blue shift of the AEDANS emission band (Fig. 7). In contrast, little or no shift is observed for the 1:1 complex with Phk5 (Fig. 7). The spectrum of the 1:1:1 ternary complex resembles that of the binary complex with Phk13 (data not shown), implying that the spatial arrangement of Phk13 in this region is not altered by the addition of Phk5.

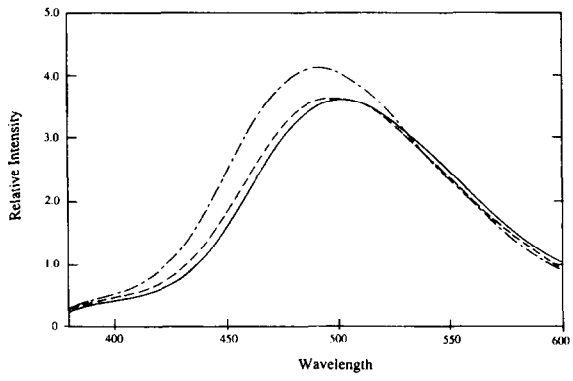

Fig. 7. Emission spectra of AED–TnC (0.8 μM) in the absence of peptide (—), in the 1:1 complex with Phk13 (– · –), and in the 1:1 complex with Phk5 (- - -). The excitation wavelength is 340 nm. Other conditions are the same as in Fig. 1.

Table 5
Transfer efficiencies and separations for binary and ternary complexes of TnC with Phk13 and Phk5 [a]

Donor	Acceptor	Complex	R_0	E	R
Trp-4	AED–Cys98	binary	19.7	0.68	17.4
Trp-16	AED–Cys98	binary	18.0	0.31	20.5
		ternary	18.0	0.16	23.7

[a] Conditions are the same as for Table 2.

A comparison of the emission spectra of Phk13w4 in the presence of excess CaM and TnC reveals no major difference in either intensity or wavelength dependence (data not shown). This is also the case for Phk5. The implication is that the microenvironments of the tryptophan groups are similar in both cases for complexes with TnC and CaM. This suggests in turn that the conformations of the two classes of complex are similar.

Examination of the emission spectra of 1:1 complexes of Phk13w4 and Phk5 with AED–TnC, in which the AEDANS group is linked to Cys-98, indicates that significant quenching occurs by radiationless energy transfer for both Trp-4 and Trp-16. However, the degree of quenching is much greater in the former case (Table 5). This is consistent with, and suggests that, Trp-4 is closer to the quenching AEDANS group, as is confirmed by calculations of the separations (Table 5).

4. Discussion

The binary complex of calmodulin and Phk13 can clearly bind more than one molecule of Phk5. This is also the case for calmodulin itself, although the effect is less pronounced [12]. However, as shown in Section 3, binding of one molecule of Phk5 is much stronger than that of subsequent molecules so that when the mole ratio of Phk5, Phk13, and CaM is 1:1:1, this is also overwhelmingly the composition of the complex. The data here have accordingly been analyzed in terms of a single Phk5 molecule in the ternary complex. It is, however, difficult to rule out some degree of localized mobility of Phk5 within the complex so that some distribution of orientations is present.

The resistance to tryptic digestion of the 1:1:1

complex, as compared with the Phk13–CaM binary complex, suggests that Phk5 is located at a position intermediate to the N- and C-terminal lobes of calmodulin, so as to hinder access to bond 77–78, which is exposed to tryptic hydrolysis in the binary complex. It remains uncertain whether the protection of this bond arises from steric interference or indirectly from an induced conformational change. The 1:1 Phk5–CaM complex is likewise partially shielded from tryptic digestion [12], despite the difference in conformation from the ternary complex [12].

The circular dichroism difference spectrum of Phk13 in the binary Phk13–CaM complex is not suggestive of a high degree of α-helix content, being more consistent with a β-structure or a random coil. The same is true for the difference spectrum of Phk13 plus Phk5 in the ternary complex. Some degree of α-helical content cannot be ruled out. However, this result is subject to ambiguities of interpretation in view of the uncertain contribution of alterations of the secondary structure of calmodulin.

Trp-16 of Phk5 in both its binary and ternary complexes appears to be closer to NO_2-Tyr-139 than to AED–Cys-27; a factor in this is probably the length of the chain linking the AEDANS group. The minor increase in separation from NO_2-Tyr-139 which accompanies the transition from the binary to the ternary complex is consistent with an extension of calmodulin upon combining with Phk13. A surprising finding is the large magnitude of the standard deviation of the distribution of separations in both cases, which is more pronounced than in the case of Phk13w4. This might reflect some distribution of orientations of Phk5 in both complexes as discussed above.

To the extent that the results for TnC are applicable to the CaM complexes, there is no evidence for a major conformational change of Phk13 upon going from the binary to the ternary complex. This is consistent with the similar effects of addition of Phk13 upon the emission spectra of AED–Cys-98 of TnC or AED–Cys-27 of CaMwG in the absence or presence of combined Phk5. This is also consistent with conclusions from low-angle X-ray scattering [11], which indicate that the ternary complex is similar in overall geometry to the binary complex with Phk13. It would also imply that Phk13 in the ternary, as well as the binary complex, has predominantly an extended β- or random structure, which is bent into a roughly hairpin-like structure [12]. However, the situation is different for the case of Phk5, whose separation from several reference points within TnC or CaM changes significantly upon formation of the ternary complex. This is especially the case for the separation of Trp-16 from AED–Cys-98 of TnC (Table 5).

References

[1] C.B. Klee, T.H. Crouch and P.G. Richman, Ann. Rev. Biochem., 49 (1980) 489.
[2] C.B. Klee and T.C. Vanahan, Adv. Protein Chem., 35 (1982) 213.
[3] N.C. Strynadka and N.G. James, Proteins, 7 (1990) 234.
[4] H.W. Jarrett and R. Madhavan, J. Biol. Chem., 256 (1991) 362.
[5] D.K. Blumenthal, K. Takio, A.M. Edelman, H. Charbonneau, K. Titani, K.A. Walsh and E. Krebs, Proc. Natl. Acad. Sci. USA, 82 (1985) 3187.
[6] P.J. Kennelly, A.M. Edelman, D.K. Blumenthal and E.G. Krebs, J. Biol. Chem., 262 (1987) 11958.
[7] M. Ikura, G.M. Clore, A.M. Gronenborn, G. Zhu, C.B. Klee and A. Bax, Science, 256 (1992) 632.
[8] W.E. Meador, A.R. Means and F.A. Quiocho, Science, 257 (1992) 1251.
[9] D.B. Heidorn, P.A. Seeger, S.E. Rokop, D.K. Blumenthal, A.R. Means, J. Creepi and J. Trewhella, Biochemistry, 28 (1989) 6757.
[10] M. Dasgupta, T. Honeycutt and D.K. Blumenthal, J. Biol. Chem., 264 (1989) 17156.
[11] J. Trewhella, D.K. Blumenthal, S.E. Rokop and P.A. Seeger, Biochemistry, 29 (1990) 9316.
[12] A. Juminaga, S.A. Albaugh and R.F. Steiner, J. Biol. Chem., 269 (1994) 1660.
[13] D.M. Watterson, F. Sharif and T.C. Vanaman, J. Biol. Chem., 255 (1980) 962.
[14] G.M. Strasburg, M. Hogan, W. Birmachu, D.D. Thomas and C. Louis, J. Biol. Chem., 263 (1988) 542.
[15] P.G. Richman and C.B. Klee, Biochemistry, 17 (1978) 928.
[16] J. Lan, S. Albaugh and R.F. Steiner, Biochemistry, 28 (1989) 7380.
[17] H.C. Cheung, C.K. Wang and F. Garland, Biochemistry, 21 (1982) 5135.
[18] T. Forster, Ann. Physik., 2 (1948) 55.
[19] L. Stryer and R.P. Haugland, Proc. Natl. Acad. Sci. USA, 58 (1967) 719.
[20] H.C. Cheung, in J.R. Lakowicz (Ed.), Topics in Fluorescence Spectroscopy, Vol. 2, Plenum Press, New York, 1991, p. 128.
[21] J.R. Lakowicz, M.L. Johnson, W. Wiczk, A. Bhat and R.F. Steiner, Chem. Phys. Lett., 138 (1987) 587.

[22] E. Haas, M. Wilchek, E. Katchalski-Katzir and I.Z. Steinberg, Proc. Natl. Acad. Sci. USA, 72 (1975) 1807.
[23] S. Albaugh and R.F. Steiner, J. Phys. Chem., 93 (1989) 8013.
[24] E. Nenortas and D. Beckett, Anal. Biochem., 222 (1994) 366.
[25] C.G. Caday and R.F. Steiner, Biochem. Biophys. Res. Commun., 135 (1986) 419.
[26] R.F. Steiner and L. Norris, Arch. Biochem. Biophys., 254 (1987) 342.
[27] S. Albaugh, J. Lan and R.F. Steiner, Biophys. Chem., 33 (1989) 71.