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Studies on the interaction of the dye, Stains-all, with individual calcium-binding domains of calmodulin

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We show that the calcium-mimic dye, Stains-all, is a convenient probe to study the structural features of the individual calcium-binding sites of calmodulin (CaM) and related calcium-binding proteins (CaBP). These peptides bind the dye in their calcium-binding sites, and induce a circular dichroism (CD) band in the bound dye in the 620 nm (J band) region, which is abolished upon the addition of calcium. Replacement of Asp by Asn in the $+x$ position of the weaker calcium-binding site (site I of CaM) abolishes the dye binding, while the same change in the higher affinity site IV attenuates the binding of the dye and does not abolish it. Replacement of Tyr in site IV with Trp does not distort the geometry, although it increases the dye binding affinity.

Calcium-binding domain; Calmodulin EF-hand motif; Stains-all; Circular dichroism

1. INTRODUCTION

The binding of calcium to calmodulin (CaM), troponin C (TnC) and the other members of the calcium-binding proteins (CaBP) is of utmost interest in the understanding of the role of calcium in a number of physiological events. It has been shown, from earlier studies of Kretsinger, that most of the calcium-binding sites in these proteins comprise a helix-loop-helix motif, which is referred to as an EF-hand motif [1,2]. A number of peptides corresponding to the calcium binding sites of various proteins have been chemically synthesized and their calcium-binding properties have been studied using several chemical and physical methods in order to understand the structural features of their binding domains [3–8]. The interaction of these peptides with other ions, such as terbium, and with cationic phenothiazine drugs, such as chlorpromazine and trifluoperazine has also been studied [9–16].

A number of indirect methods for probing the calcium-binding sites have been developed. The methods available for studying calcium binding are tedious and less sensitive, especially with proteins that have low affinities to calcium. One of the most sensitive and useful probes is the cationic carbocyanine dye, Stains-all [17]. The binding of Stains-all provides a simple and sensitive method for probing calcium binding. This dye has been shown to bind to calcium-binding sites and to generate characteristic absorption peaks and induced circular dichroism (CD) signals [18,19]. One of the features of this dye's binding is the ability of calcium to displace the dye from its binding site. Some CaBP, such as CaM, TnC and protein S100, generate both the J (620 nm) and Γ (500–510 nm) bands of the dye. Some other proteins (parvalbumin and β-crystallin) generate only the J band, while proteins like δ-crystallin generate only the Γ band of the dye. This differential behaviour is now known to reflect the structure and the conformation of the dye-binding region of the protein [20,21]. A binding region that is helical or rod-like in shape generates the Γ band of Stains-all, whereas a globular conformation generates the J band near 630 nm. This feature of the dye could thus be used for examining and comparing the affinity of calcium-binding sites in peptides and proteins, as well as their conformation.

We show here how Stains-all can be used as a ready and convenient probe to investigate the structural and conformational features, and the sequence-dependent affinity of the calcium-binding loops of the EF-hand motifs in CaBP. To this end, we have synthesized and studied some variants of the individual calcium binding loops of CaM and have focused our attention on (i) the behaviour of the individual sites with Stains-all, (ii) the contributions that coordinating and non-coordinating amino acid residues of the loop make toward Stains-all affinity, and (iii) the effect of Stains-all on the far-UV CD of the CaM peptides.

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2. EXPERIMENTAL

Stains-all and MOPS were purchased from Sigma. All other chemicals used were of analytical grade.

2.1. *Peptide synthesis and purification*

The four calcium-binding sites of CaM with the extensions towards the N-terminal (helix-loop) or only the loop region, and various substitutions in the E helix or in the loop region. were selected (Table I). Peptides were synthesized by Merrifield's solid phase method [22] with some modifications, as described earlier [23]. Crude peptides were purified by reverse-phase HPLC and their purity and identity confirmed by analytical HPLC and by amino acid sequencing.

2.2. *CD spectroscopy*

CD spectra were recorded on a JASCO J-20 spectropolarimeter. The far-UV CD spectra of the dye–peptide complexes in 2 mM MOPS, pH 7.2, in the presence of 30% ethylene glycol were recorded using a cell of pathlength of 1 cm.

2.3. *Stains-all binding assay*

All solutions used for Stains-all binding were prepared in 2 mM MOPS, pH 7.2, containing 30% ethylene glycol [18]. The molecular ellipticities were determined according to dye concentrations using the molar extinction coefficient of the dye in 100% ethylene glycol ($\varepsilon = 1.13 \times 10^5$). As far as possible the experiments were carried out in the dark so as to avoid photodamage to the dye. Increasing amounts of the substrate solution were added to aliquots of the dye as a method of titration of the dye. For comparison, identical concentrations of the dye (about 10 μM) were chosen from freshly prepared stock.

3. RESULTS AND DISCUSSION

We have studied the interaction of Stains-all with seven different peptides corresponding to the four calcium-binding sites of CaM, namely fragments of site I (residues 10–35), site II (residues 52–69), site III (residues 77–105) and site IV (112–143), and also some specific variants of a low-affinity site (site I) and of a high-affinity site (site IV) (Table I). The use of the dye, Stains-all, as an excellent reporter of the calcium-binding propensity of CaM and related proteins has been established earlier [17–21].

3.1. *CD in the visible region*

3.1.1. Site I

Fig. 1A shows that the synthetic peptide 1, corresponding to the sequence 10–35 of CaM, binds Stains-all and induces optical activity in the 610 nm region (the J band) of the dye, as a positive peptide induced CD band of ellipticity of about + 640 deg · cm^2/decimol (Table II). Of interest is the behaviour of peptide 1a (D20N), which does not induce the CD band of the dye. This substitution abolishes the ability of peptide 1a to bind the dye. This is in accord with Szebenyi and Moffat's proposal [24] that the residue, Asp, at the $+x$ position (of the octahedron coordinating the calcium ion) is critical in promoting the proper fold of the N-terminal half of the binding loop. A loop that does not have Asp at the $+x$ position, such as in cardiac TnC [25], T4 phage lysozyme [26] or δ-crystallin [27,28] binds calcium with low affinity. Our results with peptide 1a show that this inability is reflected even in the dye-binding behavior. That Stains-all binds in the calcium- binding region of the peptide is verified by the fact that addition of calcium to the peptide–dye complex reduces the CD band induced in the dye absorption region as seen in Fig. 1A.

Table I

Four synthesized calmodulin EF-hand motifs and their variants

Peptide	Amino acid sequence
	10 35
1	AEFKEAFSLFDKDGDGTITTKELGTV
1a	AEFKEAFSLFNKDGDGTITTKELGTV
	52 69
2	INEVDADGNGTIDFPEFL
	77 105
3	KDTDSEEEIREAFRVFDKDGNGYISAAEL
	112 143
4	LGEKLTDEEVDEMIREADIDGDGQVNYEEFVQ
4a	LGEKLTDEEVDEMIREADIDGDGQVNWEEFVQ
4b	LGEKLTDEEVDEMIREANIDGDGQVNYEQFVQ

Bold letters indicate the substitutions made. Underlined sequences are the actual loop regions.

3.1.2. Site II

The synthetic fragment 52–69, corresponding to site II, appears to induce a higher intensity of the CD signal in the J band region of the bound dye, in comparison to that seen with peptide 1 (Fig. 1B). Here again the addition of $CaCl_2$ displaces the bound dye and the CD band of the dye is lost, indicating that calcium successfully competes for the same site on the peptide. The CD signal is negative in sign when the dye binds to peptide II. All other peptides induce a positive CD signal in the J band region of the bound dye.

3.1.3. Site III

Reid has studied the 33-residue sequence 81–113 of

Table II

Spectral and binding parameters for Stains-all binding with calmodulin peptides

Peptides	Nature of the J band (± nm)	Normalized molar ellipticity at a dye:peptide ratio of 10
1	+ 610	+ 640
1a	*	-
2	− 620	+ 70,000
3	+ 615	+ 42,000
4	+ 615	+ 200,000
4a	+ 615	+ 703,000
4b	+ 615	+ 93,500

*Peptide 1a does not induce metachromasia.

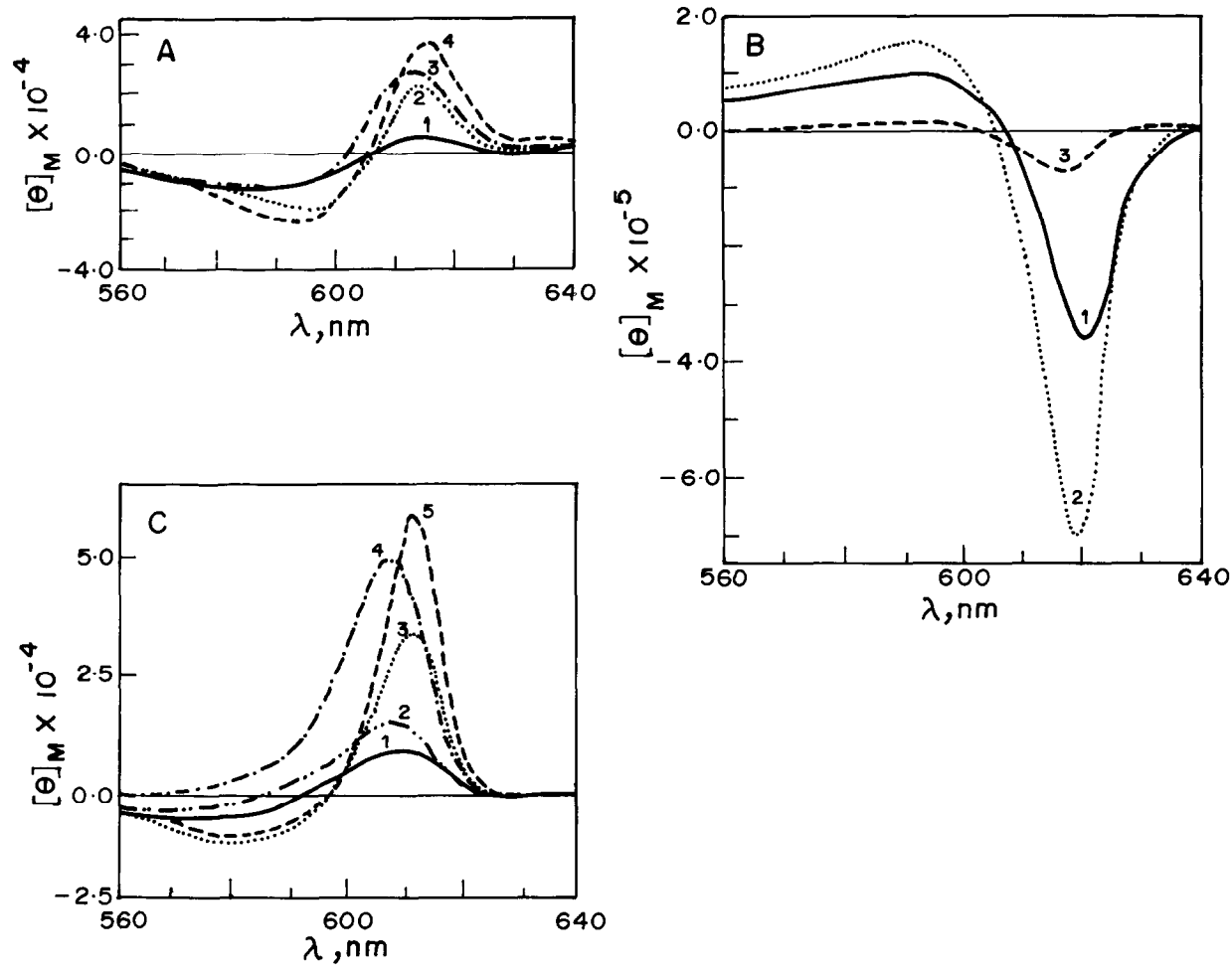

Fig. 1. CD spectra of the complex of the dye, Stains-all, with CaM peptides. The concentration of the dye used was 10 μM, and CD spectra were recorded in 1 cm pathlength cell. (A) with peptide 1: curves 1, 2, and 4 are for dye:peptide molar ratio 0.6, 0.3, and 0.16, respectively; curve 3 is obtained upon the addition of 2 mM $CaCl_2$ to the mixture representing curve 4. (B) with peptide 2: dye:peptide molar ratios in curves 1 and 2 are 4 and 2, respectively; curve 3 is obtained when 2 mM calcium was added to the mixture representing curve 1. (C) with peptide 3: dye:peptide molar ratios in curves 1, 3 and 5 are 21, 10 and 7, respectively; curves 2 and 4 are obtained when 30 mM and 15 mM calcium, respectively, was added to the mixture representing curve 3.

CaM which does not have an 'apical acid pair', although it does have the aromatic residue tyrosine in the $-y$ coordinating site [29]. We have synthesized the sequence 77–105 of this site (peptide 3, Table I) which contains 16 residues in the E helix fragment, the usual 12 residues in the loop sequence and just 1 residue following it, representing the 'helix-loop' part of the motif. This peptide induces the positive J band of the dye (Table II). Whereas initial addition of calcium to peptide 3 increases the ellipticity with about a 5 nm blue shift, further addition of calcium displaces the dye and reduces its CD band intensity (Fig. 1C).

3.1.4. Site IV

Site IV of CaM is the strongest of the four in binding calcium. We have synthesized and studied three different fragments corresponding to this calcium-binding site of CaM (Table I). Peptide 4 (residues 112–143) has the acid pair, Asp and Glu, in the $+z$ and $-z$ positions. The $-y$ position here is not a hydrophobic one but has the residue Gln.

Fig. 2A shows that, of all the sites, it is peptide 4 that generates the most intense CD of Stains-all with an ellipiticity of about 2×10^5 deg · cm^2/ decimol (Table II). Addition of calcium initially increases the J band intensity of the dye–peptide complex, indicating a conformational readjustment, as also evidenced by far-UV CD of the peptide–calcium complex, but further addition of calcium displaces the dye (Fig. 2B). Fig. 3A shows that a 4-fold increment occurs in the CD band intensity in the visible region when Stains-all binds to peptide 4a (Y138W) as compared to peptide 4, which differs in just one non-liganding residue, Trp, in place of Tyr-138. In this case, a comparatively higher concentration of calcium is required to displace the dye completely from the peptide (Fig. 2C).

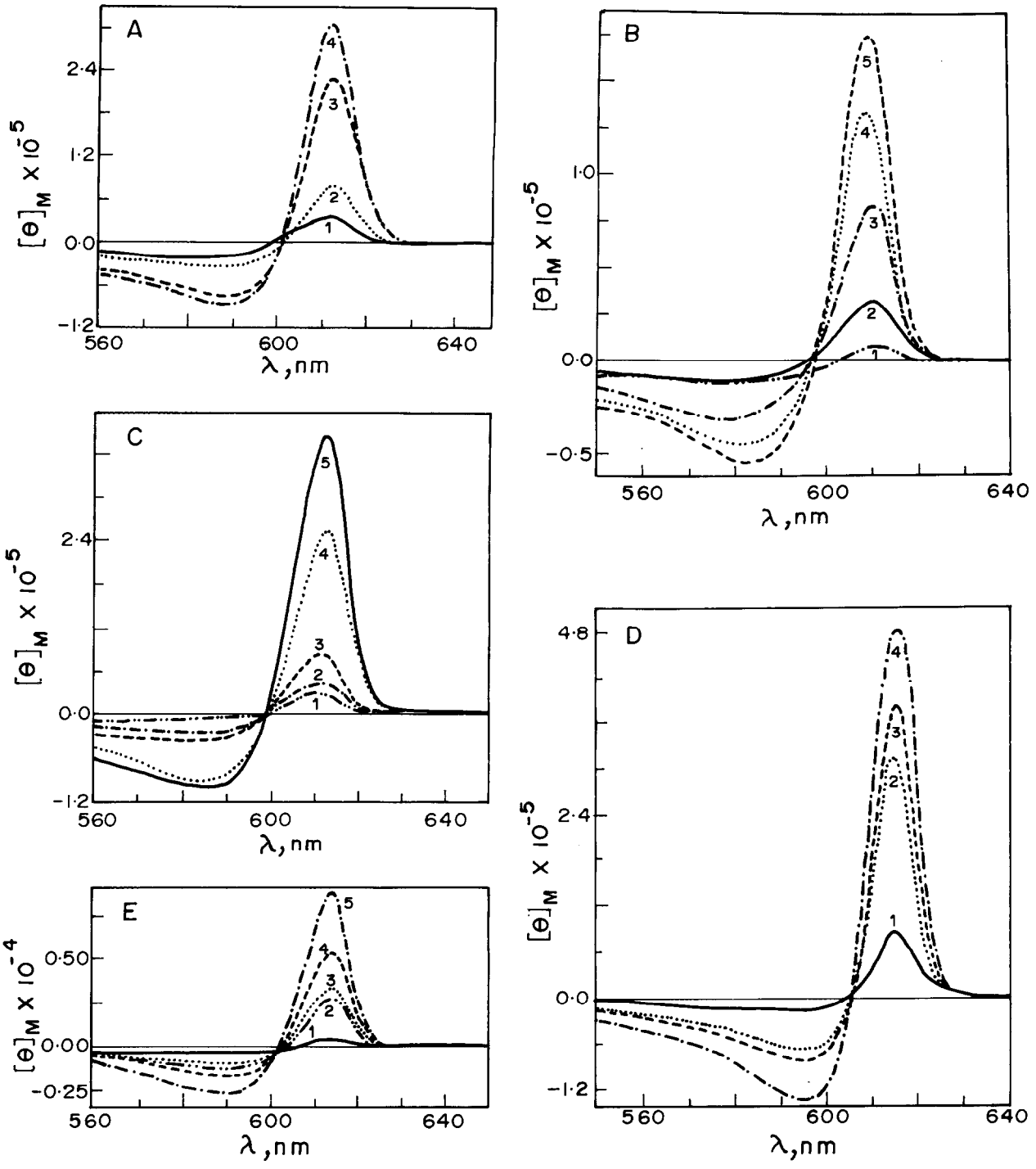

Fig. 2. CD spectra of Stains-all complex with the CaM peptides. (A) with peptide 4: the dye:protein molar ratios in the curves 1, 2, 3 and 4 are 30, 20, 10 and 7, respectively. (B) The effect of $CaCl_2$ on CD spectra of the dye–peptide 4 complex; curves 4, 5, 3 and 1 are obtained when 0.6, 1.5, 3.0 and 6 mM $CaCl_2$, respectively, was added to the mixture of curve 2 with a dye:peptide ratio of 12. (C) Effect of $CaCl_2$ on CD spectra of dye–peptide 4a complex. Dye:peptide molar ratio was 35; curves 5, 4, 3, 2 and 1 are obtained when 0, 30, 60, 100 and 150 mM $CaCl_2$, respectively, was added to the mixture of dye–peptide representing curve 5. (D) with peptide 4a: the dye:protein molar ratios in curves 1, 2, 3 and 4 are 118, 58, 38 and 29, respectively. (E) with peptide 4b: the dye:protein molar ratios in curves 3, 4 and 5 are 24, 15 and 11, respectively; curves 1 and 2 are obtained when 1.5 and 0.6 mM $CaCl_2$ was added to the mixture representing curve 3.

The effect of introducing the Trp residue in the binding site is of interest. None of the four CaBP TnC, CaM, carp parvalbumin and intestinal calcium-binding proteins, have a Trp residue in their sequence. In order to explore the intrinsic spectroscopic properties of Trp, a number of mutated CaMs with indistinguishable cal-

cium-binding properties, with tryptophans at F99W, T26W, T62W, S81W and Q135W, have been prepared [30,31]. Based on energy minimization studies, Chabbert et al. [31] observed that the substitution of Trp for the 7th residue in each EF-hand does not distort the calcium-binding geometry and does not interfere with coordination of the main chain C=O of this residue to calcium ion. Our results are in accord with these, and suggest that Trp in the non-liganding position (10th residue in the motif) enhances the dye-binding ability of the peptide. Molecular dynamics calculations on these peptides that we performed with a Macro Model program, energy-minimized using the Amber force field, showed no significant change in their geometry.

Peptide 4b has the Asn and Gln at $+x$ and $-z$ position for Asp-129 and Glu-140, respectively, but, as Fig. 2E shows, it is able to bind Stains-all and generate a CD band that is about one-third as strong as with peptide 4 ($+9 \times 10^4$ deg · cm^2/decimol, Table II), and calcium replaces the dye from the peptide. The 'EF-hand test rules' suggest that a peptide devoid of the two important amino acids, Asp and Glu, at $+x$ and $-z$ positions, respectively, should not bind calcium ions [24] and hence, expectedly, the dye Stains-all. While this is what we observe in the case of site I (peptide 1a with D20N), site IV appears to behave somewhat differently (peptide 4b). It is known that calcium affinities of the sites in the C-terminal domain are, on average, 6-fold higher than those of the N-terminal domain [32,33]. This may be the reason why peptide 4b continues to display dye-binding ability, albeit weaker than the canonical peptide 4.

3.2. *Far-UV CD*

In addition, it is worth noting that peptide 4 not only binds the dye, but does so in such an intimate manner as to induce optical activity in the 235 nm absorption band of the dye. Peptides from no other sites, that were used here, could generate this induced CD band efficiently. Furthermore, addition of calcium ions abolishes the 235 nm CD band and generates the typical CD spectrum of the Ca-bound peptide.

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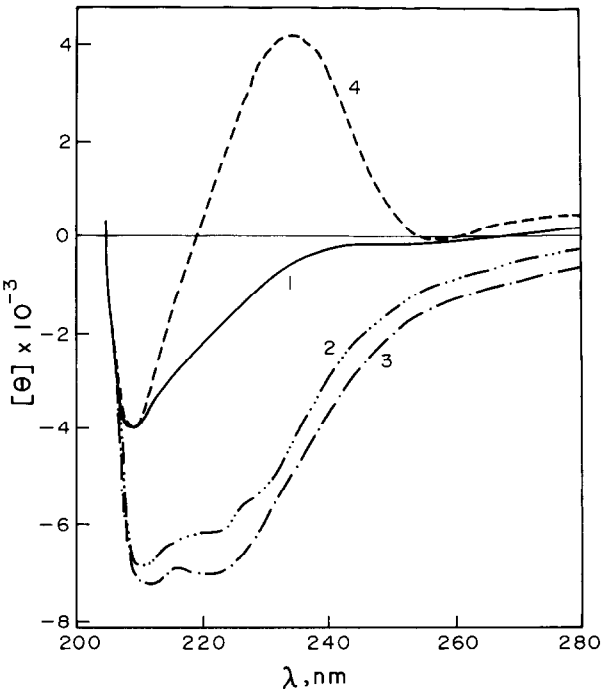

Fig. 3. Far-UV CD spectra of the dye–peptide 4 complex. Curves 1 and 4 represent the addition of 0 and 3 μM of dye to peptide 4 having concentration of 1.2 μg/ml. Curve 2 was obtained after the addition of the 20 mM calcium into the mixture of curve 4. Curve 3 shows the effect of calcium (20 mM) on the far-UV CD spectrum of the peptide 4 (curve 1) only.

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