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The conformation of calmodulin: a substantial environmentally sensitive helical transition in Ca_4-calmodulin with potential mechanistic function

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The conformation of Ca_4-calmodulin in solution, as assessed by far-UV peptide circular dichroism, contains significantly less α-helix than the proposed X-ray crystal structure. We now show that Ca_4-calmodulin adopts significant additional helical structure in solution in the presence of a helicogenic solvent (50%, v/v, aqueous 2,2,2-trifluoroethanol or 50%, v/v, methylpentane-5,5-diol). We suggest that the long continuous helix (residues 66–92 of the crystal structure) is not necessarily a normal feature of the calmodulin structure in solution, and may be due in part to the conditions of crystallisation. This result is supported by time-resolved tyrosine fluorescence anisotropy studies indicating that Ca_4-calmodulin in solution is an essentially compact globular structure which undergoes isotropic rotational motion. We conclude that, under appropriate ionic and apolar environmental conditions, Ca_4-calmodulin undergoes a substantial helical transition, which may involve residues in the central region of the molecule. Such a transition could have an important function in determining specificity and affinity in interactions of calmodulin with different target sequences of Ca^{2+}-dependent regulatory enzymes.

Calmodulin; Helical content; Circular dichroism; Conformation; Fluorescence anisotropy; Rotational correlation

1. INTRODUCTION

Although much is known about the binding of Ca^{2+} to calmodulin as the first step in the calcium-mediated regulation of a number of enzymes (review [1]), relatively little information is available at the molecular level of the mechanism of the activation process of such enzymes by calmodulin. The crystal structure at 3 Å resolution of the Ca_4-calmodulin complex [2,3] confirms the presence within the 148-residue sequence of 4 'E-F-hands' as calcium-binding sites, where the helices E and F each typically comprise about 10 helical residues [5]. These binding sites are arranged in pairs, in two globular domains of diameter 20 Å. The total helical content of the crystal structure of calmodulin is ~65% (i.e. 96 residues) [2]. The crystal structure also shows residues 66–92 in a long helix linking the two domains, giving a prolate ellipsoidal shape, of length 65 Å and axial ratio 3:1. These dumb-bell features broadly resemble those of troponin C at 2.8 Å resolution [6,7].

The conformation of calmodulin in solution has been extensively studied by optical and NMR techniques. Circular dichroism assessments (review [4]) show a helical content for Ca_4-calmodulin of 45–48% (i.e. 67–71 residues). Further, the helical content of Ca_4-calmodulin in aqueous solution is the residue-weighted sum of that of the tryptic fragments Ca_2-TR1C (residues 1–77) and Ca_2-TR2C (residues 78–148) [4]. Since the cleavage residue Lys-77 is in the middle of the

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Abbreviations: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TFE, 2,2,2-trifluoroethanol

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putative helix 66–92, and given the relative instability of short helices under normal aqueous conditions, the C-terminal sequence 66–78 of TR1C and the N-terminal sequence 79–92 of TR2C would be unlikely to adopt fully helical conformations. Although helices D (66–74) and E (84–92) are contained within the sequence 66–92, the question arises as to whether the continuous helix from residue 66 to 92 is present in Ca_4-calmodulin itself, in aqueous solution.

Several other closely related proteins [5] appear to show significantly less helical structure by CD than estimated from their crystal structures (e.g. calbindin [8–10], carp parvalbumin [11–13] and troponin C [5,6,14]). In addition to the problem of defining the exact limits of helical structure within these proteins, it is interesting that in parvalbumin, one of the best refined structures [12,13], certain helices are seen to deviate significantly from the ideal hydrogen bonding of the α-helix conformation, and appear intermediate between α- and the 3_{10}-helix. Given the sensitivity of the CD of peptide n-π^* transitions to local geometry ([15] and Manning and Woody, unpublished), such deviations could potentially affect CD intensities significantly.

2. EXPERIMENTAL

Bovine testis calmodulin was kindly provided by Professor S. Forsen (University of Lund, Sweden). All chemicals were of the highest purity commercially available and solutions were prepared as in [4].

CD spectra were recorded digitally from 260 to 185 nm using a Jasco J-600 spectropolarimeter with an instrumental time constant of 4 s. Values of $\Delta\epsilon$ were calculated using a mean residue weight of 112.7. Reported spectra were recorded at 22°C using 1 or 0.1 mm cuvettes. The buffer contained 10 mM Mes plus calcium (or EGTA) and organic solvent as required.

Time-resolved fluorescence emission (>300 nm) from excitation at 280 nm (± 2 nm) with instrumental response 224 ps FWHM, channel width 20.4 ps, peak counts 50000, count rate 3 kHz, was measured at station HA12, Synchrotron Source, Daresbury. Anisotropy was recorded from 10 cycles of 100 s alternating for each polarisation component. Calmodulin (1 mg/ml, 60 μM) in 20 mM Pipes buffer, pH 7.0, T = 20°C, was made 100 μM in EGTA; the calcium complex was made by addition of 500 μM $CaCl_2$ to this solution.

Steady-state fluorescence anisotropy was measured with an SLM-8000 spectrofluorimeter with excitation at 285 ± 2 nm and emission at 320 ± 2 nm for calmodulin solutions (0.1 mg/ml) at 20°C in semi-micro fluorescence cuvettes. Corrections for light scattering were negligible and were omitted.

3. RESULTS

Since helical structure can readily be induced in unstructured peptides by the addition of organic solvents such as trifluoroethanol (TFE), we have investigated their effect on calmodulin conformation, using UV peptide CD. Fig.1 shows that, at pH ranging from 5 to 7, 50% (v/v) aqueous trifluoroethanol induces a marked increase in helicity in Ca_4-calmodulin, with half-maximum effect at 20% TFE. Aqueous methylpentane-5,5-diol (50%, v/v) at pH 5 induces comparable CD changes; these conditions parallel those used for crystallisation [2]. A similar enhancement also occurs with apo-calmodulin at pH 7 (fig.1C). Apo-calmodulin itself undergoes a small but significant increase in CD intensity between pH 7 and 5, although Ca_4-calmodulin shows no such effect.

The magnitude of the enhanced CD in 50% TFE strongly suggests the adoption of additional helical conformation by a significant number of residues of the protein. Analysis from 191 to 240 nm shows that the total helix induced by these solvents is 60–65% (method of Provencher and Glockner [16]). This corresponds closely with the maximum helical content estimated from the X-ray structure, including the long continuous helix (66–92) linking the C-terminal helix of site II with the N-terminal helix of site III. These results suggest that the total helix content observed in the crystal structure is not normally present in calmodulin in aqueous solution but the additional secondary structure may be induced by the conditions of crystallisation.

In several properties (e.g. CD [4], Ca^{2+} dissociation rates [17], NMR [18–21]), calmodulin behaves as the sum of the two half molecules, with relatively little perturbation between side chains of residues located in the two globular domains of Ca_2-TR1C and Ca_2-TR2C when combined in Ca_4-calmodulin. However, chemical modification of Lys-75 [22], Lys-77, Met-71, -72 and -76, and proteolytic sensitivity in the central sequence [1], are all modified by the binding of Ca^{2+}, suggesting a Ca^{2+}-dependent influence of the domain conformation on the properties of residues in the linking sequence.

Changes in hydrodynamic properties when calmodulin binds calcium have been reviewed [1]. Time-resolved fluorescence studies of a non-

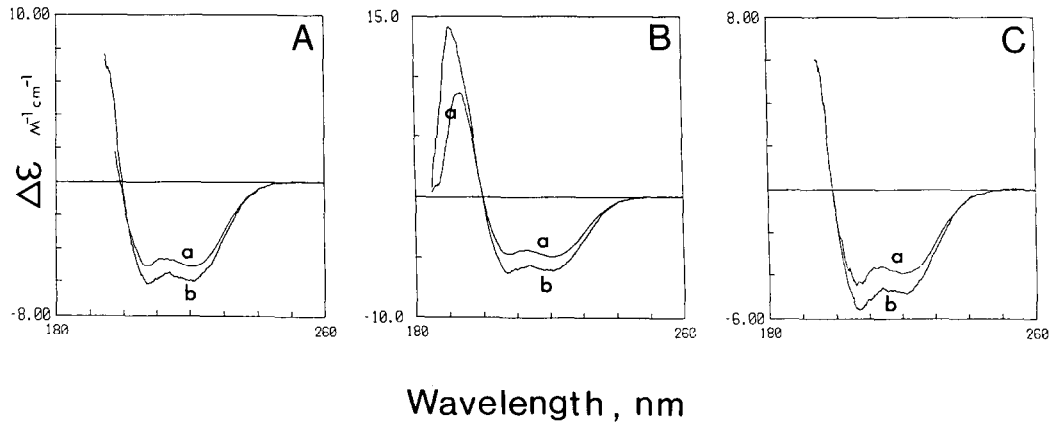

Fig.1. Effect of TFE (50%) on the far-ultraviolet circular dichroism spectrum of calmodulin. CD spectra were recorded in 10 mM Mes (a) and in 10 mM Mes plus 50% TFE (b). (A) Ca_4-calmodulin at pH 7.0; (B) Ca_4-calmodulin at pH 5.0; (C) apo-calmodulin at pH 7.0. Note the differences in scale of CD intensity.

covalent complex of Ca_4-calmodulin with TNS [23] were interpreted in terms of the 3 : 1 prolate model, and involving internal or segmental motion. However, other physical evidence is not readily compatible with the extended prolate structure. Low-angle X-ray scattering [24] gave a radius of gyration of 21.5 Å (and 20.6 Å for apo-calmodulin), apparently consistent with the X-ray crystallographic model, but the derived vector length distribution function has been questioned as being insufficiently bimodal for the dumb-bell model [25]. A more compact 'bent' model has been proposed [26].

We have addressed the problem of hydrodynamic shape by measuring the rotational correlation time of calmodulin, using the time-resolved fluorescence anisotropy [27] of the intrinsic tyrosine residues. Fig.2 and table 1 show that the fluorescence lifetime distribution of calmodulin changes significantly on binding excess Ca^{2+} (or Tb^{3+}), consistent with steady-state fluorescence data [28,29]. The total time-resolved emission of Tyr-99 and Tyr-138 in apo-, Ca_4- and Tb_4-calmodulin is well represented by a 3-exponent fit. The time-resolved tyrosine anisotropy of apo-calmodulin is only poorly fitted by a single exponent ($\chi^2 = 2.91$), but is well fitted by two processes, with $\phi_1 = 0.4$ ns and $\phi_2 = 5.6$ ns. The faster process indicates internal segmental motion close to Tyr-99 (site III) and/or Tyr-138 (site IV). By contrast, Ca_4-calmodulin (and Tb_4-calmodulin) show a single rotational relaxation time of 5.3–6.0 ns (fig.2b,right; cf. [31]). The observed value is significantly lower than those derived less directly from Perrin plots of tyrosine steady-state fluorescence data [29], or from time resolved fluorescence of non-covalent TNS-calmodulin complexes [24].

Hydrodynamic theory shows that a prolate ellipsoid with axial ratio 3 : 1 would exhibit complex anisotropic Brownian rotational diffusion [33,34], and hence in general show multiple components in fluorescence anisotropy. The observation of a single rotational correlation time indicates a relatively compact spherical hydrodynamic volume, and its magnitude is close to the value of 6.6 ns calculated for a hydrated globular protein of M_r 16800 in aqueous solution at 20°C (cf. [32]). The observed results for apo-calmodulin suggest that there is significant amplitude of segmental motion involving tyrosine (approx. ± 30° on the wobbling-in-cone model [35]). However, when calmodulin forms the complex with Ca^{2+} (or Tb^{3+}), this internal motion is inhibited, and the tyrosine residues experience the essentially isotropic global motion of the whole calmodulin molecule.

The steady-state fluorescence anisotropy of Ca_4-calmodulin is $\langle r \rangle = 0.18$ at pH 7.1, consistent with $\phi = 5.6$ ns, and $\langle \tau \rangle = 2.7$ ns (with $r_o = 0.25$ [30]). In 50% (v/v) TFE the fluorescence emission is increased 3-fold, and $\langle r \rangle = 0.11$–0.12 (for pH 4.8–7.1); the decrease in $\langle r \rangle$ is due to viscosity and lifetime effects. These results indicate the absence

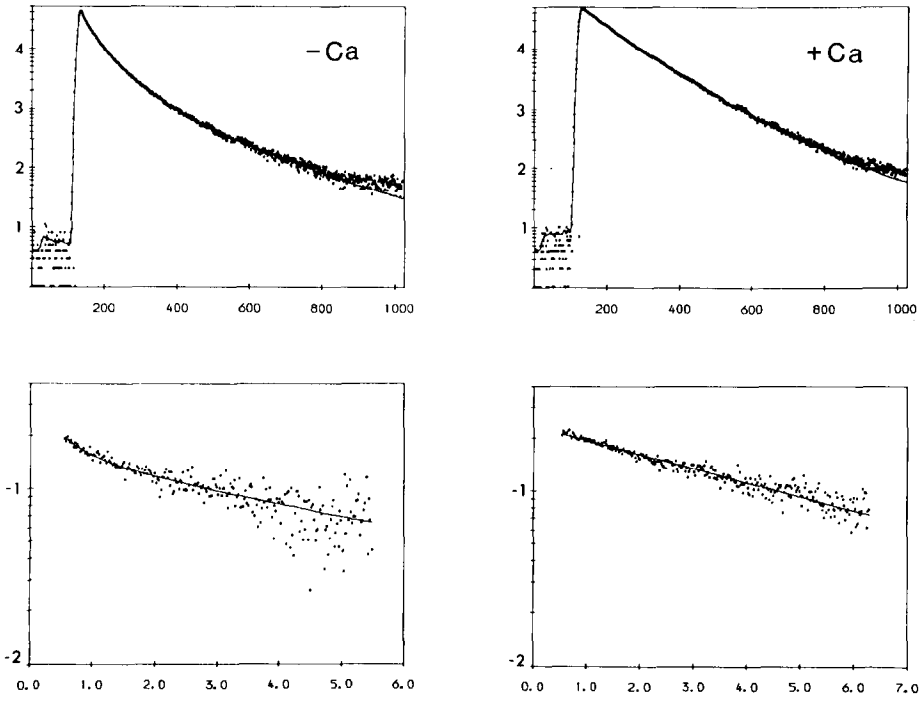

Fig.2. Time-resolved fluorescence properties of apo-calmodulin (left) and Ca_4-calmodulin (right): (top) total fluorescence intensity (log_{10}) vs channel; (bottom) fluorescence anisotropy (log_{10}) vs time (ns). The continuous lines are the computed fitted curves (see table 1). Experimental details are given in the text.

of a major increase in hydrodynamic volume in calmodulin (which would increase $\langle r \rangle$) when the helical structural transition occurs in the presence of TFE.

Table 1

Time resolved fluorescence analysis

Lifetime analysis (n = 3 components)

Sample	t_1	a_1	t_2	a_2	t_3	a_3	χ^2
+ EGTA	0.29	0.082	1.17	0.067	3.73	0.007	1.55
+ Ca^{2+}	0.15	0.046	1.59	0.074	2.92	0.043	1.37
+ Tb^{3+}	0.62	0.051	1.89	0.064	3.94	0.013	1.51

Anisotropy analysis

Sample	n	ϕ_1	r_1	ϕ_2	r_2	χ^2
+ EGTA	1			4.00	0.193	2.91
	2	0.40	0.110	5.60	0.147	1.05
+ Ca^{2+}	1			5.30	0.221	1.05
+ Tb^{3+}	1			6.00	0.192	1.03

Analysis was by standard non-linear least-squares routines for sums of exponential functions with pulse deconvolution (lifetimes) and independent fitting of sum and difference curves for anisotropy (cf. [27])

4. DISCUSSION

The time-resolved fluorescence anisotropy results indicate that Ca_4-calmodulin exists in solution as a relatively compact globular structure. It undergoes a transition with appropriate ionic and environmental conditions, to a significantly enhanced helical state, but without apparent change in hydrodynamic volume. These results cannot identify unambiguously which residues are involved in the helical change. The CD enhancement could derive from adjustment or extension of pre-existing helices, or from the initiation of new helical structure, although the scope for the latter process in Ca_4-calmodulin appears to be rather limited. A likely candidate is the central portion of the helix 66–92 which is not well defined in the crystal structure. However, it would need to be discontinuous in this enhanced helical solution conformation, in order to maintain a more compact hydrodynamic volume [27], possibly stabilised by limited domain interactions.

Although the enhanced helix has been

demonstrated under apolar solvent conditions, a transition into additional helical structure, for example to encompass residues 66–92 as proposed, could be an important component of the binding of Ca_4-calmodulin with a target enzyme. Conformational requirements of the calmodulin-binding site on individual target proteins are not known, although several binding sequences have been reported [36,37]. Interaction of the isolated target peptides with Ca_4-calmodulin indicates that substantial extra helical conformation develops on complex formation. Likewise, in other model systems, CD [38] and NMR [39] studies show that the peptide mellitin adopts a helical conformation on binding to Ca_4-calmodulin, as does mastoparan [40]. Although helix formation is not an essential prerequisite for binding to Ca_4-calmodulin (or troponin C, as suggested by the troponin-I inhibitory peptide (104–115) [41]) it appears quite common. In these model studies, the major conformational adjustment appears to be in the target peptide [36–39], but this may be the result of the small size of target peptides used. With calmodulin-binding peptide P13 from skeletal muscle myosin light chain kinase [36], major conformational changes occur in both components. These were interpreted as being a substantial secondary structure (helical) transformation in P13, and probably tertiary structure changes in Ca_4-calmodulin, but increased helical conformation in calmodulin would be entirely consistent with the observed CD effects.

The relative inefficiency of the calmodulin fragments TR1C and TR2C, either singly or together, to substitute for calmodulin in certain activating processes [42] also suggests that structural features of the linking region may be important. Specific interactions, rather than a general hydrophobic surface effect, could be important in determining complex formation. In addition to the steric advantage of the two domains being linked covalently, the capability for helical conformation of residues in the structural linkage between the domains could have a functional role in the more potent activation dependent on the binding of the whole calmodulin molecule.

Thus several subclasses of calmodulin-target complexes may exist in which both Ca_4-calmodulin and/or the target sequence undergo increases in helical conformation in forming the complex. In fact, helix formation is an important component in the conformational changes of calmodulin on binding calcium, together with a projected movement together of the helices in exposing the hydrophobic binding residues [42]. The ability of movements of helices to transmit conformational information over substantial distances was noted in the structure of troponin C [43]. We propose a simple and effective extension of this principle, namely that a given calcium regulator protein may adopt distinct conformations in complexes with different enzymes. Different helical states in the calcium-binding regulator protein, modulated by specific ionic and hydrophobic interactions induced by calcium binding, may provide discrimination in terms of the specificity and affinity of the interactions in different classes of regulator-target enzyme complexes.

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