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The effects of Ca^{2+} and Cd^{2+} on the secondary and tertiary structure of bovine testis calmodulin

A circular-dichroism study

Stephen R. MARTIN* and Peter M. BAYLEY
Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

Near-u.v. and far-u.v. c.d. spectra of bovine testis calmodulin and its tryptic fragments (TR_1C, *N*-terminal half, residues 1–77, and TR_2C, *C*-terminal half, residues 78–148) were recorded in metal-ion-free buffer and in the presence of saturating concentrations of Ca^{2+} or Cd^{2+} under a range of different solvent conditions. The results show the following: (1) if there is any interaction between the *N*-terminal and *C*-terminal halves of calmodulin, it has no apparent effect on the secondary or tertiary structure of either half; (2) the conformational changes induced by Ca^{2+} or Cd^{2+} are substantially greater in TR_2C than they are in TR_1C; (3) the presence of Ca^{2+} or Cd^{2+} confers considerable stability with respect to urea-induced denaturation, both for the whole molecule and for either of the tryptic fragments; (4) a thermally induced transition occurs in whole calmodulin at temperatures substantially below the temperature of major thermal unfolding, both in the presence and in the absence of added metal ion; (5) the effects of Cd^{2+} are identical with those of Ca^{2+} under all conditions studied.

INTRODUCTION

The heat- and acid-stable protein calmodulin mediates the effects of the intracellular messenger Ca^{2+}. Calmodulin usually interacts with its receptor protein only after Ca^{2+} is bound (Klee, 1980; Klee *et al.*, 1980). Although it is clear that the binding of Ca^{2+} causes large conformational changes in the protein (Klee, 1980), there is still considerable uncertainty with regard to the sequence of binding to the four Ca^{2+}-binding sites and also the absolute values of the association constants. However, extensive n.m.r. studies (Forsén *et al.*, 1980, 1983; Andersson *et al.*, 1982) suggest that the Ca^{2+}-binding sites can be grouped into two sites with high affinity and two with low affinity.

Limited tryptic cleavage of calmodulin in the presence of saturating amounts of Ca^{2+} results in cleavage at lysine-77, thus giving rise to two peptides of equal size (Walsh *et al.*, 1977; Drabikowski *et al.*, 1977, 1982). N.m.r. studies (Andersson *et al.*, 1983) have shown that TR_1C (*N*-terminal half, residues 1–77) contains the two weak Ca^{2+}-binding sites whereas TR_2C (*C*-terminal half, residues 78–148) contains the two strong Ca^{2+}-binding sites. This assignment has been confirmed by stopped-flow studies (Martin *et al.*, 1985).

Recent reports have indicated that Cd^{2+} is a convenient and reliable probe in the study of the Ca^{2+}-binding sites in several different Ca^{2+}-binding proteins (for review see Vogel *et al.*, 1983*a*).

There have been a number of reports on the effects of Ca^{2+} on the c.d. of calmodulin, but they show considerable variability. Here we aim to resolve some of the conflicting data in the literature. We report a comparison of the effects of Ca^{2+} and Cd^{2+} on the secondary and tertiary structure of bovine testis calmodulin and its tryptic fragments, as well as the effect of these metal ions on the stability of calmodulin towards heat- and urea-induced denaturation.

METHODS

Bovine testis calmodulin and its tryptic fragments were prepared as described elsewhere (Andersson *et al.*, 1983; Vogel *et al.*, 1983*b*). The purity of calmodulin and the fragments was checked by both SDS/polyacrylamide-gel and agarose-gel electrophoresis. A small contamination (less than 5%) of each fragment with the other cannot be excluded, because of partly overlapping bands on both SDS/polyacrylamide-gel and agarose-gel electrophoresis (Thulin *et al.*, 1984). Ca^{2+}-free proteins were prepared by passage through a Chelex-100 column; the residual metal ion was determined by atomic absorption spectroscopy (approx. 0.05 mol of Ca^{2+}/mol of protein). Protein concentrations were determined by amino acid analysis. All other chemicals used were of analytical grade and were obtained from local suppliers. The standard buffer used in this work was 20 mM-Pipes/KOH, pH 7.0.

C.d. spectra were recorded from 310 to 255 nm or from 260 to 200 nm with a Jasco J41-C spectropolarimeter equipped with a J-DPY data processor, with sensitivity of 0.5 milidegrees/cm and with an instrumental time constant of 4 s. Fused silica cuvettes of path length 0.1, 1 or 10 mm were used as required. Reported spectra were recorded at 18 °C and represent the averages of at least four scans. The spectra are presented in terms of molar ellipticity, based on mean residue weights of 112.7 (whole calmodulin), 111.2 (TR_1C) or 114.3 (TR_2C). Near-u.v. c.d. spectra were recorded at a protein concentration in the range 3–5 mg/ml, and far-u.v. c.d. spectra were recorded with a protein concentration in the range 0.1–0.15 mg/ml (1 mm cuvette) or 1–1.5 mg/ml (0.1 mm cuvette). The sample temperature was monitored directly in the cuvette with a Comark model 3002 electronic thermometer.

The general procedure for studying the effect of added metal ions was as follows. The spectrum of the metal-ion-free protein was recorded after the addition of

* To whom correspondence should be addressed.

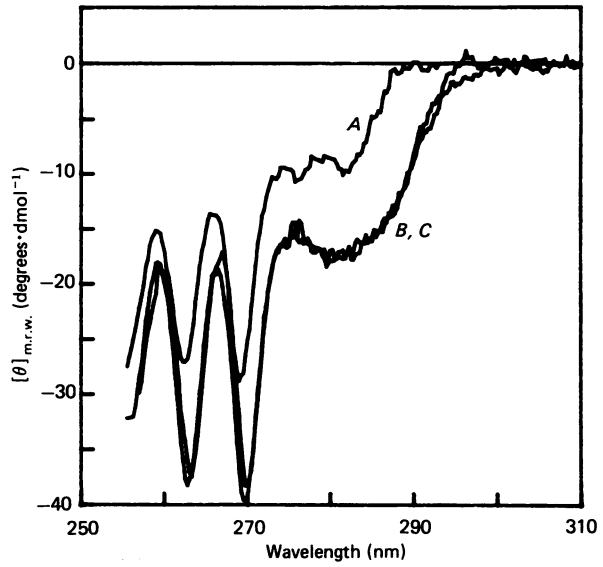

Fig. 1. Effects of Ca^{2+} and Cd^{2+} on the near-u.v. c.d. spectra of calmodulin

Spectra were recorded at 18 °C in standard buffer (20 mM-Pipes/KOH, pH 7.0). Spectrum *A*, whole calmodulin in standard buffer; spectrum *B*, plus excess Ca^{2+}; spectrum *C*, plus excess Cd^{2+}.

EDTA to a final concentration of 0.25 mM (0.10 mM for far-u.v. c.d. spectra), and the spectrum of the metal-ion-saturated form was obtained after the addition of metal ion to a final concentration of 1.5 mM for Ca^{2+} or Cd^{2+} and 5–25 mM for Mg^{2+}.

RESULTS

Near-u.v. c.d. spectra

Fig. 1 shows the near-u.v. c.d. spectra of bovine testis calmodulin in metal-ion-free buffer and with excess Ca^{2+} (and Cd^{2+}). Identical results are obtained in buffers containing 150 mM-KCl. The signal above 275 nm arises from the two tyrosine residues (positions 99 and 138, *C*-terminal half); the prominent bands at 262 and 268 nm are attributed to phenylalanine residues. The conformational change induced by binding of metal ions clearly perturbs the environment of both tyrosine and phenylalanine residues.

Figs. 2(*a*) and 2(*b*) show the effect of Ca^{2+} on the near-u.v. c.d. spectra of the tryptic fragments. As expected, TR_1C shows contributions only from phenylalanine residues, whereas TR_2C shows a spectrum qualitatively similar to that of calmodulin but with a relatively larger component from tyrosine residues. Addition of excess Ca^{2+} leads to major intensity changes from TR_2C but only to small changes for TR_1C. The addition of Cd^{2+} to the fragments gives effects indistinguishable from those shown for Ca^{2+}.

We have compared the spectrum of calmodulin with the spectrum generated by the summation for the fragment spectra (with appropriate weighting). Both in the presence and in the absence of Ca^{2+} (or Cd^{2+}) the summation is indistinguishable from the spectrum of the whole molecule (results not shown), thereby indicating that tryptic cleavage does not produce tertiary-structure changes that significantly affect the environment of the aromatic amino acid residues.

Far-u.v. c.d. spectra

Fig. 3 shows the far-u.v. c.d. spectra of bovine testis calmodulin; this Figure includes three spectra for the Ca^{2+}-free form and two for the Ca^{2+}-saturated form, to give an indication of reproducibility. The addition of Ca^{2+} produces a significant increase (approx. 21%) in the intensity of the c.d. signal. Although Cd^{2+} behaves identically, this is generally not the case for other metal ions, and we confirm that the effects induced by saturating concentrations of Mg^{2+} are substantially smaller (cf. Wolff *et al.*, 1977; Walsh *et al.*, 1979; Drabikowski *et al.*, 1982).

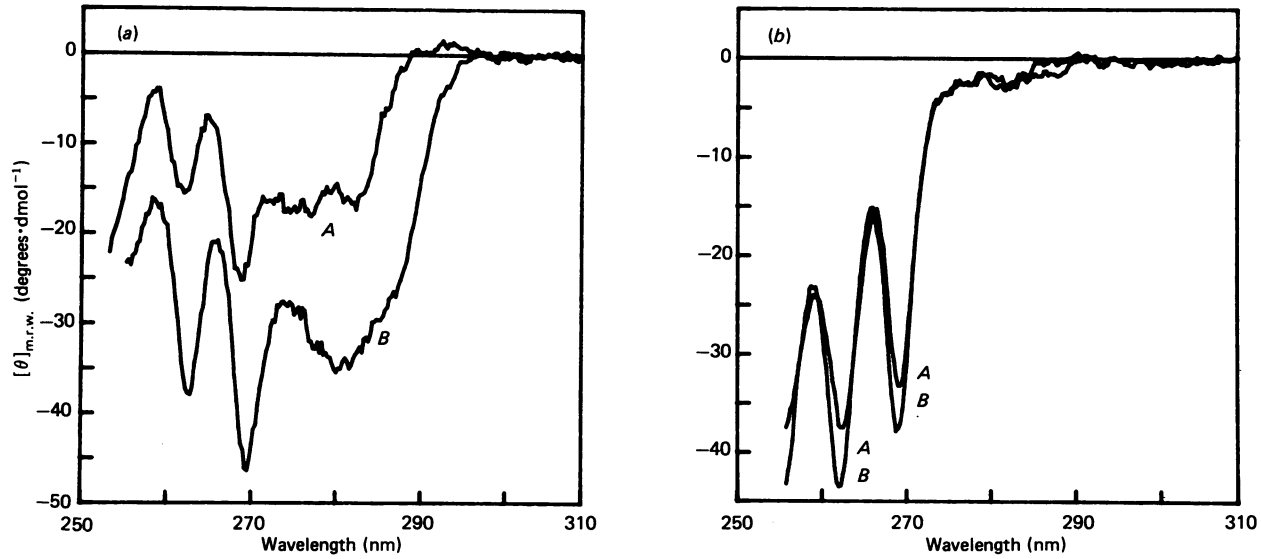

Fig. 2. Effects of Ca^{2+} on the near-u.v. c.d. spectra of tryptic fragments of calmodulin

Conditions were as indicated in Fig. 1 legend. (*a*) TR_2C in standard buffer (spectrum *A*) and plus excess Ca^{2+} (spectrum *B*). (*b*) TR_1C in standard buffer (spectrum *A*) and plus excess Ca^{2+} (spectrum *B*).

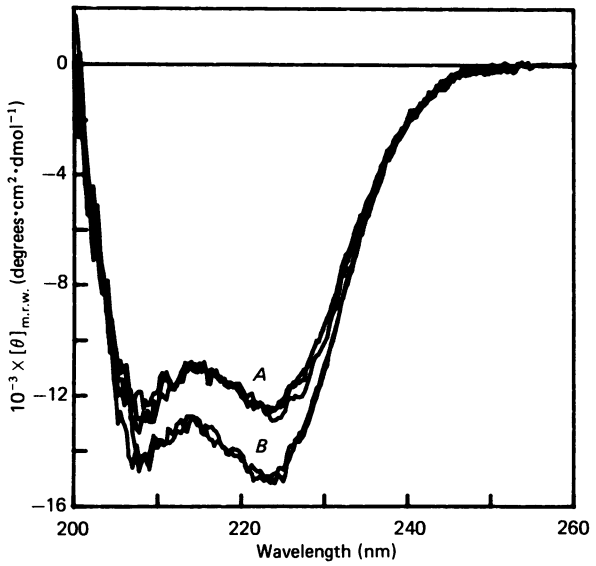

Fig. 3. Effects of Ca^{2+} and Cd^{2+} on the far-u.v. c.d. spectra of calmodulin

Spectra were recorded at 18 °C in standard buffer (20 mM-Pipes/KOH, pH 7.0). Spectrum *A*, whole calmodulin in standard buffer; spectrum *B*, plus excess Ca^{2+}.

Figs. 4(*a*) and 4(*b*) show the far-u.v. c.d. spectra of the tryptic fragments. In the absence of added metal ion, the spectrum of TR_1C is more intense than that of the whole molecule, whereas that of TR_2C is correspondingly less intense (c.f. Drabikowski *et al.*, 1982). However, in the presence of added Ca^{2+} the two fragments show spectra with almost equal intensity, which is very close to the spectrum of the whole molecule. The effect of Cd^{2+} is again identical, and the summation of the fragment spectra is identical with the spectrum of the intact molecules under all conditions.

Effects of urea

Figs. 5(*a*)–5(*d*) show c.d. intensity (222 nm) as a function of urea concentration for calmodulin (and its tryptic fragments) in metal-ion-free buffer and in the presence of saturating concentrations of Ca^{2+} (or Cd^{2+}). In agreement with previous results (Walsh *et al.*, 1979), the stability of calmodulin to urea denaturation is increased by the presence of Ca^{2+}; very similar effects are elicited by Cd^{2+}. In addition, the presence of either metal ion substantially enhances the stability of each fragment to urea denaturation. In the absence of Ca^{2+} both fragments are slightly more sensitive to urea than is calmodulin, with TR_1C being more stable than TR_2C.

Effects of temperature

Fig. 6 shows the effect of temperature on the c.d. spectrum of calmodulin over the range 10–45 °C both in the presence and in the absence of added Ca^{2+}. This temperature range is significantly below the major thermal unfolding transition of calmodulin.

DISCUSSION

Values reported for the intensity of the far-u.v. c.d. spectrum of calmodulin show considerable variability, both in the absolute intensity and in the magnitude of the change caused by Ca^{2+} binding (Table 1). Effects of pH and buffer ions do not appear to be a major contributing factor; spectra recorded in a buffer consisting of 50 mM-Tris/HCl at pH 8.0 are identical with those shown here. Although effects of salt have been reported by Crouch & Klee (1980) and by Hennessey & Johnson (1982), we find no effects of added KCl at concentrations up to 150 mM in either far-u.v. or near-u.v. c.d. spectra. By contrast, temperature is clearly a possible contributing factor, and this is discussed further below. Experimental consistency is probably a major contributing factor (cf. Hennessey & Johnson, 1982). Probable sources of error

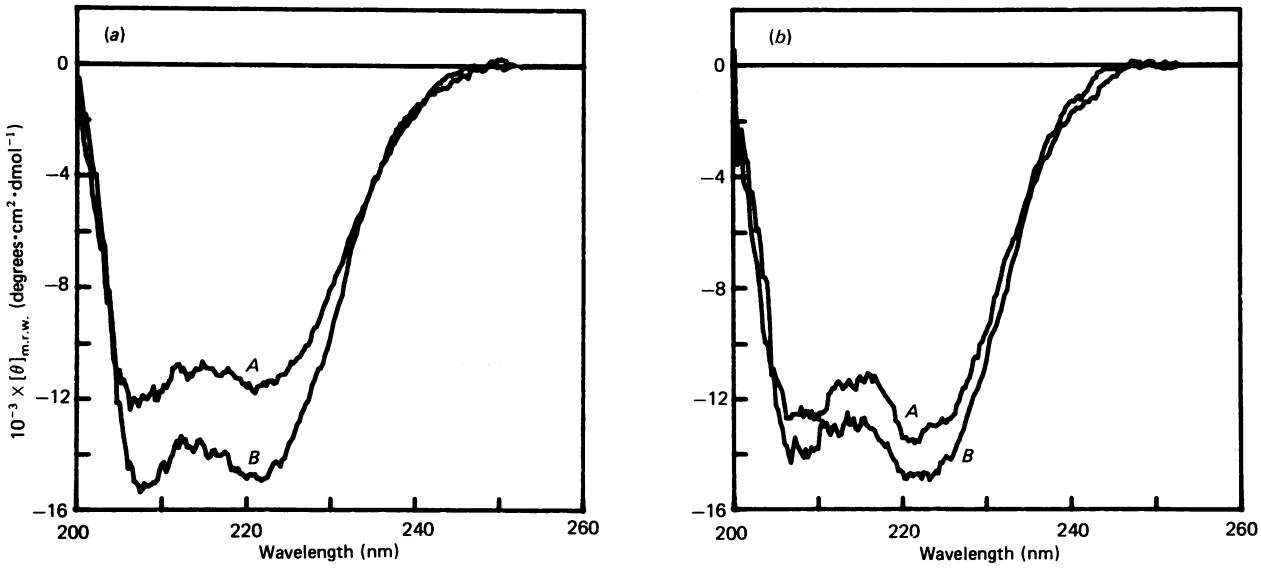

Fig. 4. Effects of Ca^{2+} on the far-u.v. c.d. spectra of tryptic fragments of calmodulin

Conditions were as indicated in Fig. 3 legend. (*a*) TR_2C in standard buffer (spectrum *A*) and plus excess Ca^{2+} (spectrum *B*). (*b*) TR_1C in standard buffer (spectrum *A*) and plus excess Ca^{2+} (spectrum *B*).

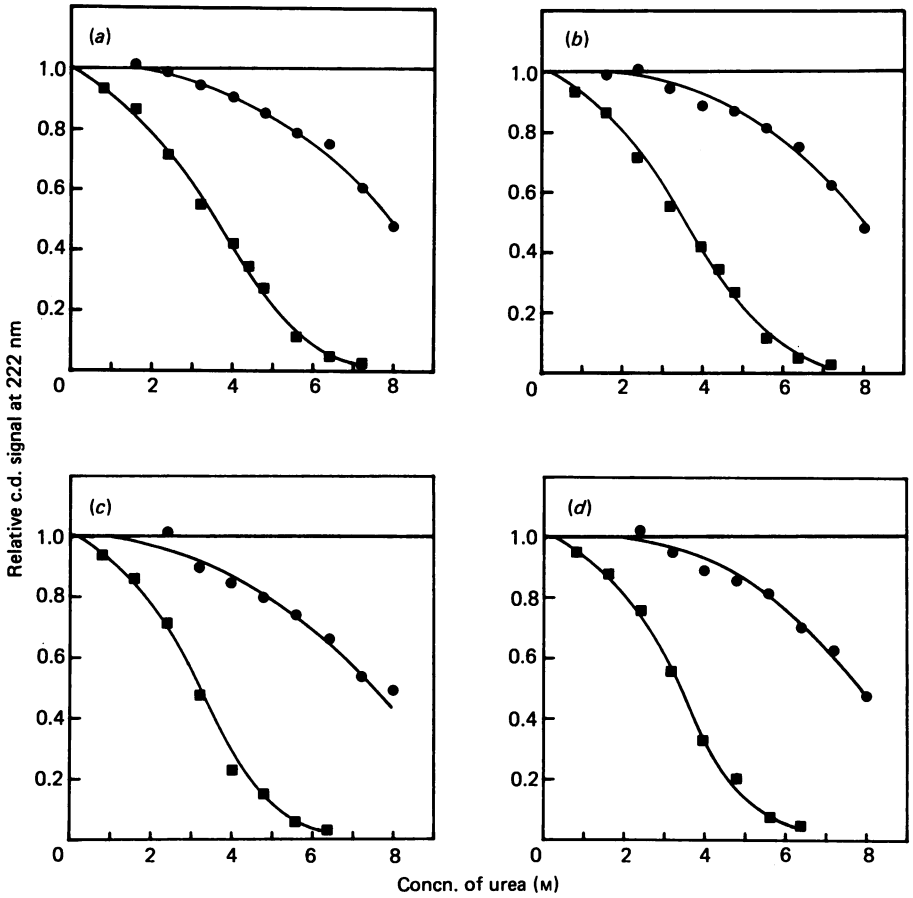

Fig. 5. Effect of urea on the relative intensity of the c.d. signal at 222 nm for calmodulin and its tryptic fragments

Spectra were recorded at 18 °C in standard buffer. (*a*) Whole calmodulin without (■) and with (●) added Ca^{2+}. (*b*) Whole calmodulin without (■) and with (●) added Cd^{2+}. (*c*) TR_1C without (■) and with (●) added Ca^{2+}. (*d*) TR_2C without (■) and with (●) added Ca^{2+}.

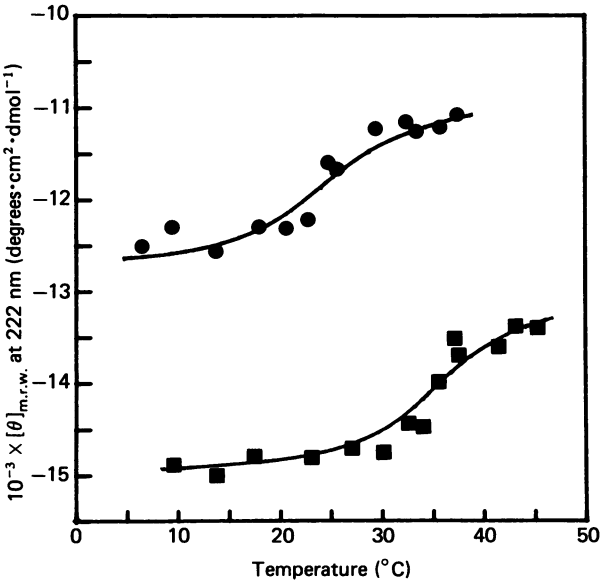

Fig. 6. Effect of temperature on the intensity of the c.d. signal at 222 nm for whole calmodulin

Spectra were recorded in standard buffer. Whole calmodulin without (●) and with (■) added Ca^{2+}.

are instrument calibration, determination of sample concentration and incorrect baseline correction procedures. We have attempted to minimize these problems by running all samples under carefully controlled conditions; use of careful instrument calibration [(+)-camphor-10-sulphonic acid] and use of amino acid analysis to determine concentration.

The far-u.v. c.d. spectra of the tryptic fragments of calmodulin are very similar to those of the whole molecule, except for minor variations in intensity. In metal-ion-free buffer the values of $[\theta]_{222}$ (means ± S.D.) are $-12300(\pm 300)$, $-13300(\pm 400)$ and -11400 (± 300) degrees·cm²·dmol⁻¹ for calmodulin, TR_1C and TR_2C respectively. These values increase by 21, 29 and 9% respectively in the presence of saturating concentrations of Ca^{2+} (or Cd^{2+}). As a consequence of the relative magnitude of these increases, the spectra of calmodulin and its fragments are very similar in the presence of added metal ion (compare Figs. 3 and 4).

The near-u.v. c.d. spectra reported here are similar to those reported by Kilhoffer *et al.* (1981), but differ substantially from those reported by other workers (see Table 2). The intensities that we find are rather higher than those generally found, the increase in intensity induced by the addition of Ca^{2+} (or Cd^{2+}) is large (cf. Walsh *et al.*, 1979) and we see no effect of added KCl

Table 1. Far-u.v. c.d. intensities of calmodulin

Without added Ca^{2+}		With added Ca^{2+}		
$[\theta]_{222}$ (degrees·cm²·dmol⁻¹)	Helix content (%)	$[\theta]_{222}$ (degrees·cm²·dmol⁻¹)	Helix content (%)	Reference
−11200	28	−15100	42	Wolff *et al.* (1977)
−15400	—	−19750	—	Drabikowski *et al.* (1982)
−14700	45	−18200	54	Dedman *et al.* (1977)
−12000	40	−14700	49	Walsh *et al.* (1979)
−12900	—	—	—	Richman & Klee (1978)
−11500	30–35	−15000	35–43	Klee (1977)
−13300	31	−15300	37	Siegel & Haug (1983)
−12200	38–40	−14800	45–48	Present work*

* Structure predictions quoted here were performed by the method of Provencher & Glockner (1981).

Table 2. Near-u.v. c.d. intensities of calmodulin

$[\theta]_{262}$ (degrees·cm²·dmol⁻¹)	$[\theta]_{268}$ (degrees·cm²·dmol⁻¹)	$[\theta]_{280}$ (degrees·cm²·dmol⁻¹)	Concn. of KCl (mM)	Reference
Without added Ca^{2+}:				
−29.6	−30.1	−9.2	150	Kilhoffer *et al.* (1981)
−14.9	−14.1	−5.2	0	Crouch & Klee (1980)
−21.9	−23.0	−6.1	100	Crouch & Klee (1980)
−20.7	−19.6	−4.2	150	Walsh *et al.* (1979)
−5.83	−4.5	+1.8	0	Wolff *et al.* (1977)*
−28.0	−30.0	−9.8	0	Present work
With added Ca^{2+}:				
−38.9	−38.7	−17.5	150	Kilhoffer *et al.* (1981)
−28.9	−31.7	−14.9	100	Crouch & Klee (1980)
−24.9	−20.3	−6.7	150	Walsh *et al.* (1979)
−16.4	−15.8	−7.2	0	Wolff *et al.* (1977)
−38.0	−40.0	−17.5	0	Present work

* The spectrum recorded by these workers in the absence of Ca^{2+} actually shows a strong positive maximum at about 273 nm with $[\theta]_{273}$ approx. 5.0 degrees·cm²·dmol⁻¹.

(150 mM) (cf. Crouch & Klee, 1980). Since the fundamental band shape is consistent, the variations probably arise from the experimental problems outlined above.

The results presented here show that, if there is any interaction between the two halves of calmodulin, it has no effect on the environment of the aromatic amino acid residues. Although this is in agreement with studies using other physical techniques, it must be emphasized that we have no information about the relative contribution of each residue to the total intensity of the c.d. spectrum. However, the high intensity per residue $\{[\theta]_{262}^{Phe}$ equals −830 (+Ca^{2+}) and −720 (−Ca^{2+}) degrees·cm²·dmol⁻¹ for TR_1C and equals −660 (+Ca^{2+}) and −270 (−Ca^{2+}) degrees·cm²·dmol⁻¹ for $TR_2C\}$ indicates that the c.d. signal probably consists of contributions from each residue (Strickland, 1974). The small effect of Ca^{2+} on the near-u.v. c.d. spectrum of TR_1C is rather surprising in view of the similar distribution of phenylalanine residues in the two fragments, but may be related to the observation that the intensity per residue of phenylalanine in the metal-ion-free form of TR_1C is already very high.

Walsh *et al.* (1979) reported that metal-ion-free calmodulin was completely unfolded by 6 M-urea whereas the Ca^{2+}-saturated form retained 60% of its structure at this urea concentration. The results presented here confirm this result for Ca^{2+} and show that added Cd^{2+} produces an identical effect. Furthermore, the stabilizing effect of Ca^{2+} and Cd^{2+} also exists for both of the tryptic fragments.

Temperature affects the conformational properties of calmodulin and its tryptic fragments at temperatures substantially below those of major thermal unfolding. [$T_{\frac{1}{2}}$ values for the major transition are 55, 49 and 45 °C in the absence of Ca^{2+} for whole calmodulin, TR_1C and TR_2C respectively, and > 90 °C for each species in the presence of Ca^{2+} (Brzeska *et al.*, 1983).] Such low-temperature effects have been noted elsewhere. Brzeska *et al.* (1983) demonstrated (for metal-ion-free calmodulin) that a plot of $d[\theta]_{222}/dT$ against T, which showed a maximum at 55 °C (major transition), showed an unexplained shoulder at 30 °C. Drabikowski *et al.* (1982) showed that an increase in temperature from 12 to 27 °C results in an 8% decrease in ellipticity at 222 nm (metal-ion-free calmodulin), and that changes in temperature affect the amplitude of the Ca^{2+}-induced changes in $[\theta]_{222}$, as confirmed here (see Fig. 6). Both Kilhoffer *et al.* (1981)

and Gangola & Pant (1983) have found discontinuities (interpreted as conformational changes) in plots of fluorescence intensity at temperatures below 40 °C; however, they disagree about the influence of Ca^{2+} on the change. Our results show that transitions in this temperature range do, in fact, occur in the presence and in the absence of added Ca^{2+}.

Under all conditions used in this work we can find no evidence that tryptic cleavage of calmodulin leads to changes in the secondary or tertiary structure of the two halves (cf. Martin *et al.*, 1985), supporting the idea that calmodulin is constructed from two largely independent domains. The effects of Cd^{2+} are identical with those of Ca^{2+}, thus confirming the view that Cd^{2+} behaves as a close analogue of Ca^{2+} (Vogel *et al.*, 1983*a*). Other metal ions generally induce changes different from those produced by Ca^{2+}; thus both Mg^{2+} and Mn^{2+} induce only small increases, and Al^{3+} causes a decrease in intensity in the far-u.v. c.d. spectrum (Siegel & Haug, 1983).

Ca^{2+} (and Cd^{2+}) clearly induce substantial conformational changes in calmodulin and its tryptic fragments. The magnitude of the change is greater for TR_2C than for TR_1C, and this may correlate with the location of the strong Ca^{2+}/Cd^{2+}-binding sites in TR_2C. The conformational change greatly increases the stability of calmodulin (and its tryptic fragments) to both urea- and temperature-induced denaturation. Finally, we have shown that calmodulin (in both the metal-ion-free and the metal-ion-saturated form) undergoes a small conformational transition at temperatures substantially below the main thermally induced transition.

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