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Evidence of noncovalent dimerization of calmodulin

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Calcium-binding proteins, such as S-100, dimerize readily, and this phenomenon plays an important role in their regulation of target enzymes [Krebs, J., Quadroni, M. & Van Eldik, L.J. (1995) *Nat. Struct. Biol.* **2**, 711–714; Kilby, P.M., Van Eldik, L.J. & Roberts, G.C. (1996) *Structure* **4**, 1041–1052]. We have investigated by Fourier-transform ion cyclotron resonance (FTICR) MS the conformational states of the calcium-binding protein calmodulin, and present clear evidence for a calmodulin dimer formed as a result of noncovalent interactions between folded monomers. Ultra-high-resolution electrospray ionization (ESI) mass spectra for calmodulin, obtained with a 9.4 T FTICR mass spectrometer, are presented. With the use of denaturing solutions (1 : 1 acetonitrile/water + 1% formic acid), relatively high charge states ($20 < z < 10$) of monomeric calmodulin ions were detected, whereas when calmodulin was electrosprayed from buffer, monomers ions with only 5–10 charges were detected. CD measurements for calmodulin in buffered solution revealed that its α-helical content was significantly higher than that for calmodulin in acetonitrile/water solutions, consistent with a proposition that changes in charge state distributions observed in the MS experiments reflect differing states of calmodulin folding.

Under buffered conditions, noncovalently bound calmodulin dimers were observed by ESI FTICR MS. Analytical ultracentrifugation experiments carried out in the same solution conditions as those used in the MS experiments were consistent with the proposed calmodulin dimer–monomer equilibrium. The ultra-high mass resolution achieved with the 9.4 T FTICR mass spectrometer allowed unequivocal identification of the noncovalent, as opposed to covalent, character of the calmodulin dimer.

Keywords: calmodulin; dimer; electrospray ionization; Fourier-transform ion cyclotron resonance (FTICR); noncovalent interactions.

An understanding of protein conformational changes is especially important with a protein such as calmodulin that is able to activate many different target enzymes [1,2]. Dimerization processes, like conformational changes, may modify the interfaces available for target protein recognition, which can, in turn, either activate or inhibit a target protein's action upon binding [3,4]. There is a growing need to develop methods to characterize protein–protein interactions. To date sedimentation equilibrium [5], X-ray crystallography [6], NMR [7] and X-ray and light scattering [8] have appeared to be the established methods for characterizing dimeric motifs. Nevertheless, dimer characterization remains quite difficult, especially when involving weak noncovalent interactions.

In recent years, electrospray ionization (ESI) MS has become important in structure–activity relationship studies. This technique allows proteins with molecular masses even greater than 100 kDa to be transported from their natural aqueous environments into the gas phase with, under appropriate conditions, the retention of primary and even higher-order structures [9–11]. It has been argued that *in vacuo* ESI mass spectra may reflect aqueous solution chemistry [12–16], and as a consequence ESI MS is beginning to be used quite widely to gain information on protein conformation and noncovalent protein–ligand binding in solution [17,18]. Fourier-transform ion cyclotron resonance (FTICR) MS is important in this context because of the exceptionally high mass resolution accessible (see [19] for a review). High-field FTICR allows mass measurements with mass resolutions of more than 100 000 and mass accuracies of better than one part per million, and, as a consequence of allowing the resolution of the isotopic components of a given species, brings a special advantage to studies of proteins and other macromolecules.

The superfamily of calcium-binding proteins is an illustrative example of how protein conformational changes serve to transduce external stimuli into a cellular response. As a result of calcium binding, calcium-binding proteins undergo conformational changes [20,21] leading specifically to conformations that activate the appropriate enzymes. Calmodulin is one of the more representative proteins of this family (for reviews see [22,23]).

After calcium activation [24,25], calmodulin is able to interact with a range of enzymes which include myosin light chain kinase, fodrin, calmodulin kinase II, phosphodiesterase and inositol 1,4,5-trisphosphate kinase 1. These enzymes are known to be involved in many physiological processes of importance, such as glycolysis, muscle contraction, cell division, cell growth, exocytosis and endocytosis. In addition, calmodulin may interact without calcium activation as is the case with neuromodulin and brush-border myosin I [26].

The structure of the calmodulin–calcium complex has been resolved by X-ray crystallography [27–29] and NMR studies [30,31], and calmodulin is shown to exhibit a dumbbell shape

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Abbreviations: ESI, electrospray ionization; FTICR, Fourier-transform ion cyclotron resonance.

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composed of two globular domains linked by a long flexible central helix [32]. Each globular domain contains two helix–loop–helix motifs or EF-hands which are expected to be the main calcium-binding sites [32,33]. Previous biophysical studies have shown that additional sites of weaker affinity are also present on calmodulin [34,35]. The structure of the bare calmodulin protein without calcium (apo-calmodulin) proved more difficult to elucidate and this was achieved, only a few years ago, by NMR [26,36,37]. X-ray-crystallographic data on apo-calmodulin protein are, as far as we are aware, not yet available.

Calmodulin is a highly flexible protein and can transitorily adopt a more extended conformation, which exposes highly hydrophobic regions. This feature is common among the calcium-binding protein superfamily and it has been suggested that these hydrophobic regions play a role in the dimerization of some calcium-binding proteins, e.g. troponin C [5].

We present here a study of dimerization of calmodulin using ESI coupled with FTICR MS. CD and ultracentrifugation experiments were performed using the same sample conditions as for MS.

EXPERIMENTAL PROCEDURES

Protein synthesis and purification

DNA-encoded calmodulin was produced by previously described procedures and purified by column chromatography [38,39]. The purity of the protein was checked by SDS/PAGE and high-pressure capillary electrophoresis and was found to be around 99%. In all experiments ultrapure water (Elga system) and plastic ware, washed in 1 M HCl, were used to minimize metal cation and other contamination. Calmodulin (4.5 mg) was dissolved in 2.5 mL of ammonium acetate (10 mM, pH 5.9) and purified over a desalting PD10 column (Pharmacia, Uppsala, Sweden), previously equilibrated with ammonium acetate. Approximately 10 fractions, of 1 mL each, were collected. Bradford reagent was used to check for the presence of the protein in each individual fraction, and showed that the last seven fractions contained protein. To avoid cation contamination, only fractions 4 and 5 were conserved and pooled. The calmodulin concentration was determined by UV absorption on a Jasco V-550 spectrophotometer using a molar absorption coefficient (ε_{280nm}) for calmodulin of 1560 $M^{-1}\cdot cm^{-1}$ [40].

FTICR MS experiments

MS measurements were made using an FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a shielded 9.4 T super-conducting magnet (Magnex Scientific Ltd, Abingdon, Oxon, UK), a cylindrical 'infinity' ICR cell with diameter 0.06 m and an external ESI source (Analytica of Branford, Branford, USA), and has been described previously [41,42]. The ESI source was equipped with a capillary made of Pyrex coated on both ends with platinum paint. The flow rate used for the ESI source was 30 $\mu L\cdot h^{-1}$; the electrospray cone voltage in the region between the nozzle and the skimmer was generally kept at low voltages. Solutions were sprayed at room temperature, and carbon dioxide, heated to a temperature of ≈150 °C, was used as the drying gas in the electrospray source. The background pressure in the ICR analyser cell was usually below 2×10^{-10} Mbar.

For the study of calmodulin under 'denaturing' conditions, electrospray solutions were prepared in water/acetonitrile (1 : 1, v/v) containing 1% formic acid with a calmodulin concentration of 50 μM. The addition of acetonitrile (or methanol) is believed to facilitate the desolvation of the droplets formed at the beginning of the electrospray process. Such solutions were found to be optimal for producing stable electrospray conditions, but of course do not mimic physiological conditions. To represent physiological conditions more closely, electrospray solutions were prepared in 10 mM ammonium acetate (using high-purity water) and buffered to the appropriate pH using acetic acid or ammonia. The concentration of calmodulin was 50 μM in all experiments, unless stated otherwise.

CD measurements

CD spectra were recorded on a Jasco J-715 spectropolarimeter, and corrected for background CD absorbance using the water/acetonitrile and ammonium acetate solutions. Concentrations of calmodulin in the solutions were determined by UV absorbance to allow accurate determination of the α-helical content.

Ultracentrifugation analysis

Sedimentation equilibrium experiments of the 'low-speed' type [43] were performed using a Beckman Optima XL-I analytical ultracentrifuge fitted with absorption and Rayleigh interference optics together with full on-line computer data capture and analysis facilities (Beckman, Palo Alto, CA, USA) [44]. Double-sector cells of 12-mm optical path length were used with a solution column of ≈1 mm (80 μL) and run at 20 °C at a rotor speed of 130 000 ***g***. A partial specific volume of 0.7235 $mL\cdot g^{-1}$ was used, calculated from the amino acid sequence according to the conserved formula of Perkins [45]. Equilibrium solute distributions were analysed using MSTARA (for absorption optics) and MSTARI (for interference optics) [44] which use the computerized M* method [46,47].

RESULTS AND DISCUSSION

'Denaturing' conditions

Figure 1A shows the ESI FTICR mass spectrum obtained for calmodulin in a 1 : 1 water/acetonitrile solution containing 1% formic acid. The mass resolution achieved for the spectrum shown in Fig. 1A exceeded 100 000 at m/z = 1500, and the intact protein ions were clearly isotopically resolved. The experimentally measured mass of the most abundant isotope, averaged over several of the observed charge states, was 16 626.85 ± 0.02 Da. Simulation of the isotope pattern calculated from the empirical formula indicated that the most abundant isotope ion would contain 10 ^{13}C atoms, so that the isotopically pure (^{12}C, ^{1}H, ^{14}N, ^{16}O, ^{32}S) calmodulin would therefore have a mass of 16 616.83 Da. This is in agreement with the theoretical mass of the isotopically pure protein (16 616.821 Da) to within 0.02 Da.

The measured charge distribution of the calmodulin ions sprayed from the water/acetonitrile solution has an approximately Gaussian shape centred at a maximum of 14 attached protons. The maximum number of charges observed ($[M + 20H]^{20+}$) exceeds the total number of basic amino acids on the protein, which is 16, assuming that only the N-terminus and lysine, arginine and histidine residues of the protein were protonated. The higher numbers of attached protons that were observed could be due to protonation of secondary amines, such as those present on the asparagine and glutamine residues.

Buffered conditions

Calmodulin solutions were prepared using ammonium acetate buffer without hydro-organic solvent (i.e. neither acetonitrile nor

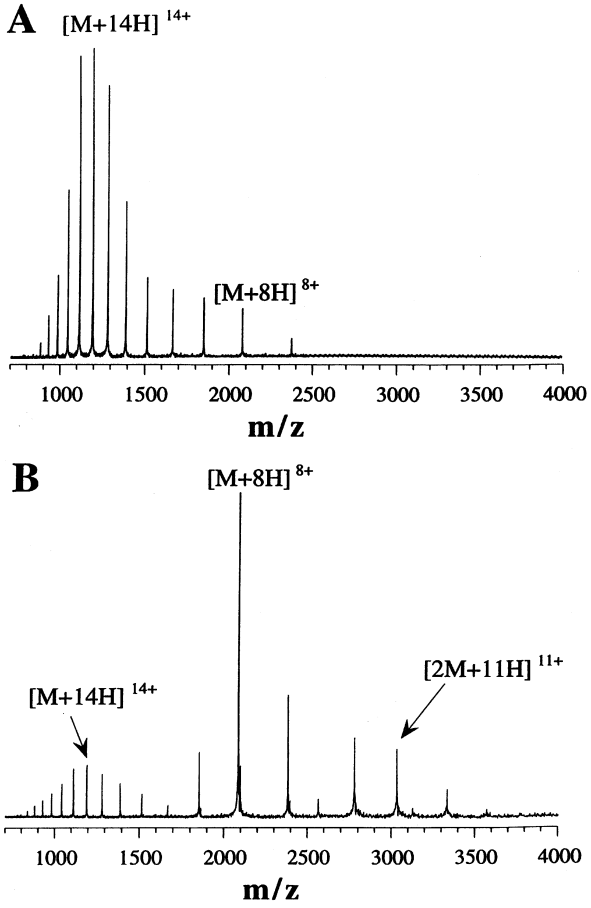

Fig. 1. ESI FTICR mass spectra of calmodulin (50 μM) in (A) 1 : 1 water/acetonitrile containing 1% formic acid and (B) ammonium acetate buffer, 10 mM, pH = 5.9. Apart from the differences in solution medium, these ESI FTICR spectra were recorded under identical experimental conditions.

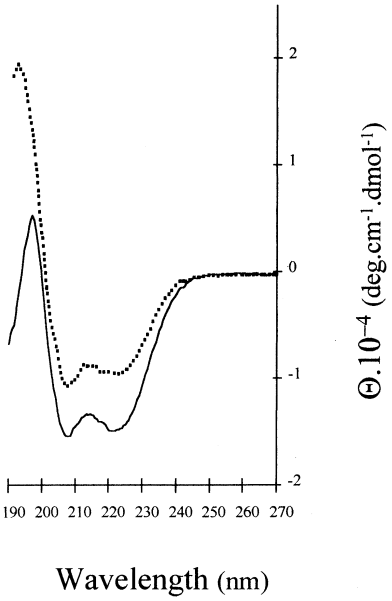

Fig. 2. CD spectra of calmodulin in 1 : 1 water/acetonitrile containing 1% formic acid (dashed line) and in ammonium acetate 10 mM (pH 5.9) (solid line).

methanol was present), in order to obtain mass spectra under more physiologically relevant conditions. As can be seen in Fig. 1B, the charge distribution in the resulting ESI spectrum was at least bimodal, suggesting the presence of at least two different protein conformations. The higher-charge part of the bimodal distribution, with a maximum of 14 attached protons, was similar to the charge distribution observed for calmodulin electrosprayed from a water/acetonitrile solution, whereas the lower-charge envelope exhibited a very strong maximum at around eight attached protons. It is expected that some of the protonatable sites in calmodulin would be involved in the formation of α-helices of folded conformations, or deeply buried inside the folding pattern, and so compact folded conformations of calmodulin might be expected to have less tendency for protonation than denatured partly unfolded calmodulin. The second envelope of the bimodal spectrum is attributed to the presence of compact folded calmodulin proteins in aqueous buffer solution. The existence of a relationship between charge state distributions in ESI mass spectra and protein conformation has been proposed for other proteins, including myoglobin [48,49], lysozyme [50–52], cytochrome *c* [12,14,51] and ubiquitin [12,52,53].

CD spectra of calmodulin were recorded in 1 : 1 water/acetonitrile containing 1% formic acid and in ammonium acetate buffer (Fig. 2). In ammonium acetate buffer, the α-helical content, calculated as described previously [54], was 41 ± 5%. In water/acetonitrile/formic acid solutions the α-helical content was 23 ± 5%, confirming that calmodulin was only partly folded in these 'denaturing' conditions. This result is consistent with CD data in the literature [55], and indicates that the ammonium acetate buffer used for these MS measurements was able to preserve a more folded calmodulin conformational state. CD spectra were also recorded in the presence of 1 mM $CaCl_2$ (data not shown). The binding of calcium to calmodulin under buffered conditions is evidenced by a small increase in the ellipticity, as expected from the rearrangement of the helices [55].

Calmodulin dimer

The ESI mass spectrum (Fig. 1B) measured under the more physiologically relevant conditions revealed the presence of a new species with *m/z* values of ≈2558, 2772 and 3024. The ultra-high mass resolution obtained with the FTICR allowed isotopic resolution of this series of ions. The individual isotope peaks of the signal at *m/z* = 3024 were spaced by 1/11 or 0.091 *m/z* units, indicating that they originated from ions with 11 charges. The mass of these ions was therefore ≈11 × 3024 = 33.26 kDa. The spacing of the isotope peaks for the *m/z* = 2772 and 2558 signals were 1/12 and 1/13, respectively, corresponding to ions with the same mass (after adjusting for the number of protons) but with 12 and 13 charges, respectively. These three peaks represent a charge state distribution pattern for the dimeric calmodulin species.

Figure 3A and B show in detail parts of the mass spectrum of calmodulin in buffer (taken from Fig. 1B), centred at *m/z* = 2772 and 3024, respectively. Figure 3A reveals the isotopically resolved mass spectra of a dimeric species $[2M + 12H]^{12+}$, which overlaps in the spectrum with the monomeric $[M + 6H]^{6+}$ species. Figure 3B shows the spectrum of a dimeric $[2M + 11H]^{11+}$ species alone. Isotopically resolved mass spectra of such large proteins at relatively low charges (and thus high *m/z* values) illustrate the mass-resolving power of high-field FTICR MS. The most abundant isotope of the dimer ions,

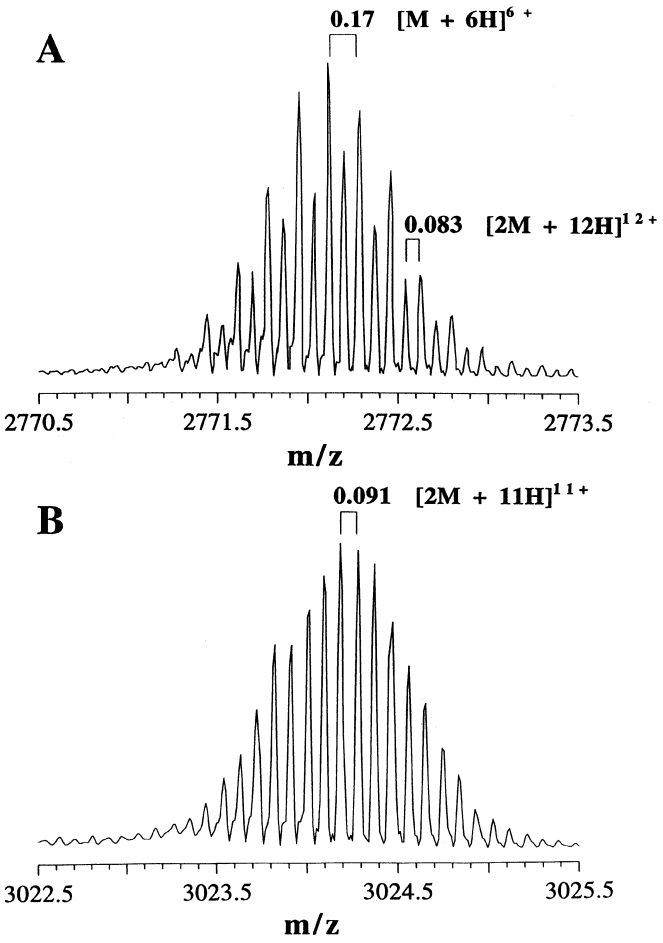

Fig. 3. Enlargements of the ESI FTICR mass spectra of calmodulin (50 μM) in ammonium acetate, 10 mM pH 5.9, showing isotopically resolved patterns. (A) Two overlapping isotope envelopes of the monomer species, $[M + 6H]^{6+}$, and the dimer species, $[2M + 12H]^{12+}$; (B) isotopically resolved mass spectrum of the calmodulin dimer with 11 protons attached, $[2M + 11H]^{11+}$.

measured over these three charge states, was 33 253.721 Da. Simulation of the spectra revealed that the dimeric ions exhibited exactly twice the mass of the calmodulin protein monomer indicating that the dimer is a noncovalently bound species. A covalently linked dimeric species with one covalent bond, such as has been shown to be formed via photoactivated dityrosine formation [56], would have a composition [2Calmodulin – 2H], and have a molecular mass 2 Da lower than measured in these experiments. It is emphasized that these FTICR measurements unambiguously distinguish species with a mass difference of 2 Da in a molecular mass of 33.2 kDa.

Consistent with the proposed presence of a noncovalent species, the relative intensities of the dimeric ions, with respect to the monomeric ions, decreased rapidly when the ESI conditions were harshened by increasing the voltage difference between the nozzle and the skimmer in the electrospray source region (data not shown). This behaviour indicates that the dimer was weakly bound and would, in itself, tend to exclude covalent bond formation as a likely dimerization pathway. No dimer species were detected in the spectra recorded by spraying calmodulin from 'denaturing' water/acetonitrile/acid solutions, i.e. conditions that normally lead to the destruction of such noncovalent interactions.

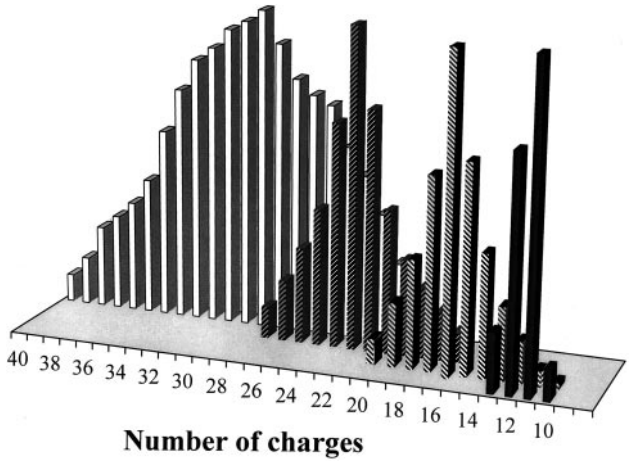

Fig. 4. Expected charge distribution patterns for calmodulin dimers composed of two unfolded monomers (open bars), one folded and one unfolded monomer (dark hatched bars), and two folded monomers (light hatched bars), compared with the experimentally observed charge distribution (solid bars).

A noncovalent calmodulin dimer possesses twice as many basic amino acids as the monomer, and so a first approximation would predict that the calmodulin dimer might bind twice as many protons as the calmodulin monomer. Figure 4 shows the results expected on this basis if the dimer was composed of two unfolded, one folded and one unfolded, or two folded monomers, when the dimer envelopes would be centred at charge states of 28, 22, or 16, respectively. The charge distribution observed for the proposed calmodulin dimer ions was a narrow approximately Gaussian envelope with a maximum at 11 attached protons. The conclusion we draw from the narrowness of this distribution is that all the observed dimer ions explore the same conformational space. The observation of only low charge states of the measured dimer ions implies that interaction between two folded monomers results in the loss of about five protonatable sites which become involved in, or hidden as a result of, the interactions between the two folded monomers to form the dimer. The loss of a higher number of protonatable sites would have to be considered if these low charge state species were a result of dimerization occurring between two unfolded or between one folded and one unfolded monomer conformation (17 and 11, respectively).

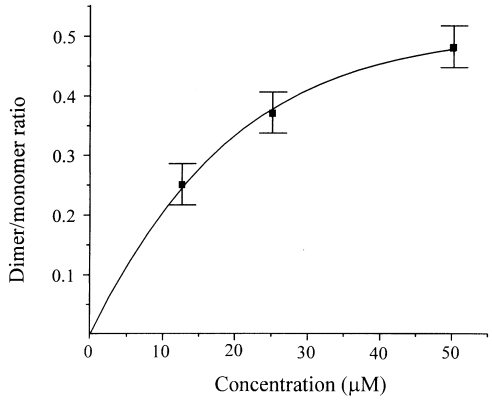

Fig. 5. Concentration dependence of the experimentally observed ratio between calmodulin dimer and calmodulin monomer determined from ESI FTICR mass spectra in ammonium acetate buffer, 10 mM (pH 5.9).

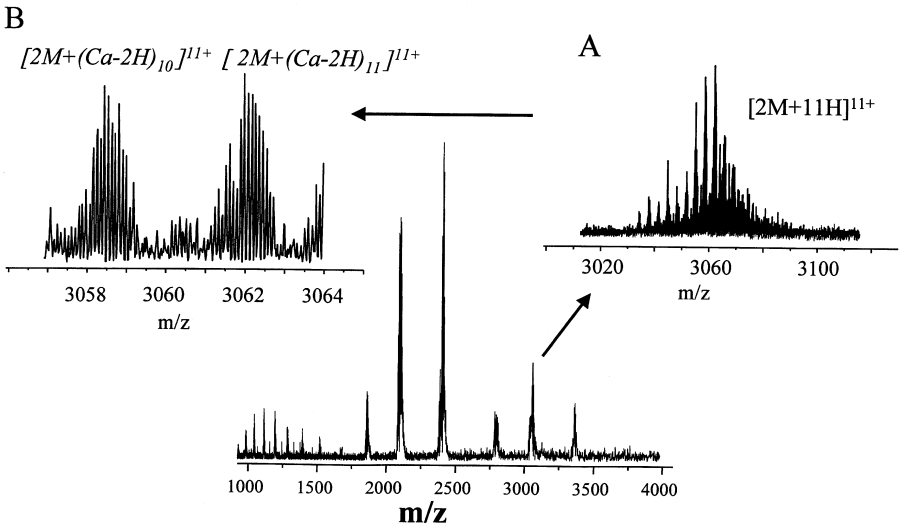

Fig. 6. ESI FTICR mass spectrum of a 25-μM solution of calmodulin in ammonium acetate, 10 mM pH 5.9, containing 1 mM $CaCl_2$. (A) Enlargement of the region from *m/z* 3020 to *m/z* 3100. (B) Enlargement of the region from *m/z* 3058 to *m/z* 3064.

Concentration dependence

Concentration-dependence experiments were undertaken to determine whether the intensities of the calmodulin dimers in the mass spectra were consistent with their formation in solution before ionization. Quantification of the change of the dimer to monomer ratios in the mass spectra with concentration was not trivial. The ratio was constant if the intensities of both the folded and unfolded monomer signals were taken into account. This result was a reflection of an observed increase in the amounts of unfolded monomer in the mass spectra and was not an indication that there was no change in the amount of dimer on increasing calmodulin concentration. Reasons for the increase in unfolded monomer intensity with concentration remain unclear. The dimer to folded monomer ratios were calculated on the basis that the calmodulin dimer was preferentially formed via noncovalent interactions between two folded monomers. The sum of the ion signals between the 6^+ and the 10^+ charge states was used as a measure of the folded monomer.

Figure 5 shows the ratio of dimer to folded monomer ions calculated from the ESI spectra, as a function of calmodulin concentration. As can be seen, the ratio of dimer ions to folded monomer ions increases over the measured calmodulin concentration range, consistent with the hypothesis that the calmodulin dimers are in equilibrium with the free monomer in solution. ESI-FTICR mass spectra were also recorded for 50 μM holo-myoglobin in buffer (molecular mass 17.6 kDa), under the same conditions as used for calmodulin, and only monomer ions were detected (data not shown). Holo-myoglobin was selected because this protein is believed not to dimerize in the solution conditions employed here.

Dimeric species of calmodulin with signals from *m/z* = 2800–3400 were also observed in the presence of 1 mM $CaCl_2$, as illustrated in Fig. 6 for a 25-μM solution of calmodulin. The masses determined from the mass spectra confirmed that these dimers were noncovalently bound. Expansion of these signals (Fig. 6A,B) revealed a series of dimeric species with 0 to at least 18 calciums bound, above which the noise level made deciphering of the signal difficult. This observation appears to be consistent with ESI results obtained for calmodulin monomer, which showed that at least 10 calciums could be attached per monomer [35]. The dimer to folded monomer ratio for a 25-μM calmodulin solution containing 1 mM $CaCl_2$ was calculated to be 0.36 ± 0.04, compared with 0.37 ± 0.04 in the absence of calcium. We therefore conclude that the presence of calcium did not change the overall dimer to folded monomer ratio, although, as expected, changes were observed in the charge state distribution patterns [57]. A detailed study of calcium-binding effects and stoichiometries for dimer and monomer species and their implications will be discussed in a future publication.

Ultracentrifugation measurements

Sedimentation-equilibrium experiments in the analytical ultracentrifuge were carried out subsequent to the MS as an independent test of the proposal of a noncovalent interaction occurring in solution. Samples were run at three concentrations (15, 30 and 60 μM), but only the loading concentration of 60 μM (≈1 $mg \cdot mL^{-1}$) gave measurable UV absorbance scans, because of the very low absorption coefficient of calmodulin (ε_{280nm} = 1560 $M^{-1} \cdot cm^{-1}$ [40]). Similarly, fringe shifts obtained from interference optics were too low for both the 15 and 30 μM sample to allow any meaningful data analysis. Results obtained for the 60 μM sample were consistent with the proposed monomer–dimer equilibrium. The apparent weight-average molecular masses, $M_{w,app}$, obtained for interference optics and absorption optics measurements were 21 400 ± 3000 Da and 21 800 ± 4000 Da, respectively.

From these data it is possible to estimate the molar dissociation constant, K_d. For a dimerizing system, correct to first-order in concentration, c ($g \cdot mL^{-1}$) [58]

$$\frac{1}{M_{w,app}} = \frac{1}{M_1} + 2\left(B_{11} - \frac{K_2}{M_1^2}\right)c \qquad (1)$$

K_2 is the dimerization constant ($mL \cdot mol^{-1}$), M_1 is the monomer molecular mass and B_{11} is the monomer–monomer second thermodynamic virial coefficient (in $mL \cdot mol \cdot g^{-2}$). If the system is assumed to be ideal, $B_{11} \approx 0$, then K_2 is simply

$$\frac{M_1^2}{2c}\left(\frac{1}{M_1} - \frac{1}{M_{w,app}}\right) \qquad (2)$$

Taking M_1 = 16 626 Da, $M_{w,app}$ = 21 600 ± 3000 Da and c = 60 μM which is equivalent to 1 $mg \cdot mol^{-1}$, K_2 is calculated to be 1.92×10^6 $mL \cdot mol^{-1}$, and K_d (equivalent to $1/K_2$) is

520 ± 70 μM, indicating a weak interaction. This value represents an upper limit for K_d: had nonideality been taken into account, a lower K_d (stronger interaction) would have been predicted.

Ionic strength and pH effects

In order to explore whether the presence of salt at physiological ionic strength inhibited dimerization of calmodulin, ultracentrifuge experiments were performed using three different KCl concentrations: 2, 50 and 150 mM. The apparent weight-average molecular masses were determined to be 18 900, 18 300 and 17 200 Da (± 1000 in each case), respectively. An increase in salt concentration therefore appeared to hinder but not exclude dimer formation.

The extent of calmodulin dimer formation was studied as a function of solution pH, where the pH was varied between 4.3 and 8.3 using acetic acid or ammonia (Fig. 7). Sedimentation-equilibrium experiments [5] have shown that skeletal troponin C, another calcium-binding protein, is a monomer at pH 7.5, but predominantly present as a dimer at pH 5.2. The ratio of dimer ions to folded monomer ions in the ESI FTICR spectra of calmodulin did not significantly change at pH 4.3, 4.9 and 5.9 and was ≈0.48 ± 0.05. Increasing the pH further to 6.9 and then to 8.3, however, led to a marked decrease in the ratio of dimer ions to monomer ions, indicating that calmodulin dimer formation was hindered under more basic conditions. The dimer to folded monomer ratio at physiological pH (7.5), was still significant at 0.3. This observation of a pH effect for calmodulin dimerization is consistent with the proposal that electrostatic interactions are important in the calmodulin dimerization process.

Electrostatic interactions within dimers formed between isolated calcium-binding loops of EF-hand proteins such as calmodulin have been observed by Wójcik *et al.* [59]. The very fact that dimerization has been shown to occur between isolated calcium-binding loops supports the proposal made here that dimerization occurs intermolecularly. Previous X-ray-scattering measurements reported by Heidorn & Trewella [60] have indicated calmodulin aggregation at pH 5.5 in the presence of Ca^{2+}, in agreement with our data. There was no X-ray-scattering evidence for aggregation without Ca^{2+} or at a higher pH, but these authors did not rule out the possible presence of some percentage of calmodulin dimer. Similarly Crouch & Klee [61] concluded from sedimentation-equilibrium experiments that calmodulin existed mostly as a monomer, but did not rule out the presence of a dimer. This lack of evidence for aggregation in the absence of Ca^{2+} might well raise an important consideration concerning the use of EGTA to chelate Ca^{2+} from solution. Could EGTA be responsible for hindering noncovalent interactions between calmodulin monomers? This question would be especially relevant if considering the importance of electrostatic, as opposed to hydrophobic, forces for calmodulin dimerization.

In negative ion electrospray mass spectra reported by Loo and coworkers [62] for bovine calmodulin there was evidence of another species with *m/z* values between those of the regular monomeric calmodulin signals, i.e. between 10^- and 9^-, and 9^- and 8^- and so on (see Fig. 2b [62]). These signals were labelled as *m/z* 1775, 1985 and 2259 and we suggest that they correspond to the dimeric form of calmodulin. Estimating the ratio of monomer to (presumed) dimer signal from their spectrum gave a value between 0.2 and 0.3 for the pH range 6.5–8, consistent with our findings.

According to the sedimentation-equilibrium results, the apparent dimerization constant for calmodulin is high (K_d = 520 ± 70 μM), i.e. the interaction between the monomers

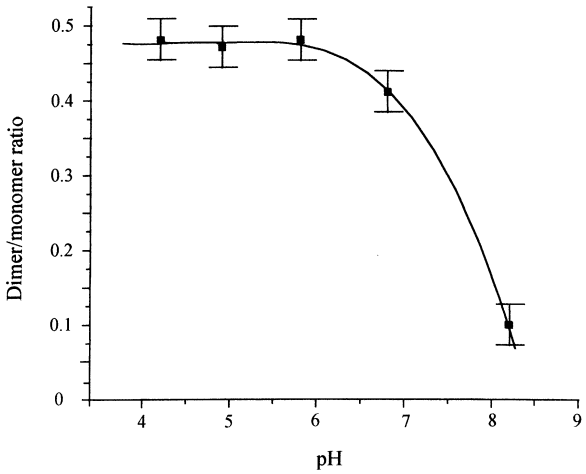

Fig. 7. pH dependence of the experimentally observed ratio between calmodulin dimer and calmodulin monomer determined from ESI FTICR mass spectra for a 50-μM solution in ammonium acetate buffer (10 mM).

is weak. Ideality is assumed for the system and this K_d value therefore represents an upper limit. In addition, the sedimentation-equilibrium experiments do not distinguish between folded and unfolded monomer, so that we might expect a lower K_d if only folded monomer was considered. Although our experiments appear to show a higher dimer to monomer ratio than would be expected from the K_d determined in solution, it is our opinion that more work is required before absolute K_d values can be deduced by MS. The accurate determination of absolute equilibrium constants by MS is still the subject of considerable debate.

The average cellular calmodulin concentration is ≈5 μM and the presence of high concentrations of salt would, according to our sedimentation results, shift the monomer–dimer equilibrium towards monomer species. Nevertheless, dimer formation, and thus dimer function, will be important within areas of the cell where the local salt and protein concentrations become favourable. These results raise a caveat for calmodulin-ligand-binding studies, especially those that are performed at low salt concentrations, namely that results will be affected by a certain monomer to dimer ratio.

Experimental characterization of calmodulin dimers has previously been difficult to achieve. ESI coupled with FTICR MS has been shown here to be an appropriate method for the identification of weakly bound calmodulin complexes present in small amounts. Analytical ultracentrifugation experiments have provided support for the existence of the calmodulin dimer–monomer equilibrium in the solution conditions used in the MS experiments. The formation of calmodulin dimer from folded monomer species with the loss of an average of five protonatable sites is proposed. The clear distinction of signals for dimer and monomer species inside the same charge state, possible because of the high resolution afforded by this technique, clearly demonstrates the power of FTICR for identification of superimposed dimer and monomer signals. Furthermore, this work highlights the potential of high-field FTICR MS for the detection and unambiguous distinction between noncovalent and covalent dimeric species (with only a 2-Da difference over a total of more than 33 000 Da in our case). Other calcium-binding proteins, for example certain S-100 proteins (data not shown), have been found to undergo both covalent and noncovalent dimerization. It is expected that ESI FTICR MS will become a valuable

complement to the established biophysical methods used to study protein aggregation.

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