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Calcium-Induced Increase in the Radius of Gyration and Maximum Dimension of Calmodulin Measured by Small-Angle X-ray Scattering†

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ABSTRACT: We have used solution small-angle X-ray scattering to characterize bovine brain calmodulin in the presence and absence of calcium. In the presence of calcium, calmodulin exists in solution as an elongated molecule with a radius of gyration of 21.5 Å and a maximum vector length of approximately 62 Å. These values are consistent with the dimensions recently determined for the crystal form of rat testis calmodulin. In the absence of calcium, the calmodulin molecule is shorter, the radius of gyration decreases to 20.6 Å, and the maximum vector length decreases to approximately 58 Å. This change in dimensions is consistent with an overall contraction of the protein through movement of the two lobes closer to each other upon removal of calcium from calmodulin.

Small-angle X-ray scattering (SAXS)[1] from macromolecules is used to measure shape-related parameters of individual molecules in free solution. As a low-resolution technique, solution scattering is insensitive to fine structural features but can accurately provide details related to size and shape. For biological macromolecules such as proteins, where interactions with substrates or regulators can change the shape, X-ray scattering provides a useful means of measuring such effects [e.g., see McDonald et al. (1979) and Pickover et al. (1979)]. If the crystal structure of a protein is known, this technique can also help determine whether the molecule exists in solution in a similar conformation to that found in the crystal. In the present study, we have used SAXS to characterize bovine brain calmodulin in solution. Calmodulin is a ubiquitous, multifunctional intracellular calcium receptor protein that belongs to a family of homologous, low molecular weight proteins which includes troponin C, parvalbumin, S-100 protein, and vitamin D dependent intestinal calcium binding protein. A three-dimensional structure has been recently determined, by X-ray crystallography, for calmodulin in which all four calcium binding sites are occupied by calcium (Babu et al., 1985). This structure shows a long, dumbbell-shaped molecule in which the calcium binding sites are arranged in two pairs, well separated by a single, long central α helix. In overall appearance, the tertiary structure is similar to that observed in the crystal structure of troponin C (Sundaralingam et al., 1985; Herzberg & James, 1985).

As with many other members of this family of proteins, calmodulin undergoes a conformational change when it binds calcium [for a review, see Klee & Vanaman (1982)]. Its α-helix content increases, and a new hydrophobic surface appears which has been implicated in the calcium-dependent interactions of calmodulin with its target proteins, peptides, and phenothiazine drugs. In addition, proteolytic fragmentation, chemical modification, and electrophoretic migration patterns change considerably when calmodulin binds calcium. Some of the structural changes are probably localized near the sites of calcium binding and may be confined to each lobe of the molecule. The magnitude of many of these changes, involving rearrangements of parts of the polypeptide chain or of individual groups of atoms, would preclude SAXS detection. However, as noted by Babu et al. (1985) and Sundaralingam & Rao (1985), the additional possibility exists, in both calmodulin and troponin C, that movement of the two lobes relative to each other occurs on binding calcium. SAXS has proven extremely useful in detecting changes on this level.

Here we describe studies using small-angle X-ray scattering to measure the size and shape parameters of calmodulin in solution and to determine how the binding of calcium to the molecule changes these parameters.

Experimental Procedures

Materials. Calmodulin was prepared from bovine brain by the method of Masure et al. (1984). Calmodulin-dependent phosphodiesterase was prepared from bovine brain as described by Head et al. (1979). Hen egg-white lysozyme (grade I), EGTA, and MOPS were obtained from Sigma.

Sample Preparation. Calmodulin with calcium was prepared by dialyzing protein for at least 24 h at 4 °C against 5 mM $CaCl_2$, 100 mM KCl, and 50 mM MOPS–KOH, pH 7.4, using washed and rinsed Spectrapor 1 dialysis tubing (molecular weight cutoff = 8000).

Calmodulin free of calcium was prepared by exhaustively dialyzing protein against 300–500 volumes of 5 mM EGTA,

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[1] Abbreviations: SAXS, small-angle X-ray scattering; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate.

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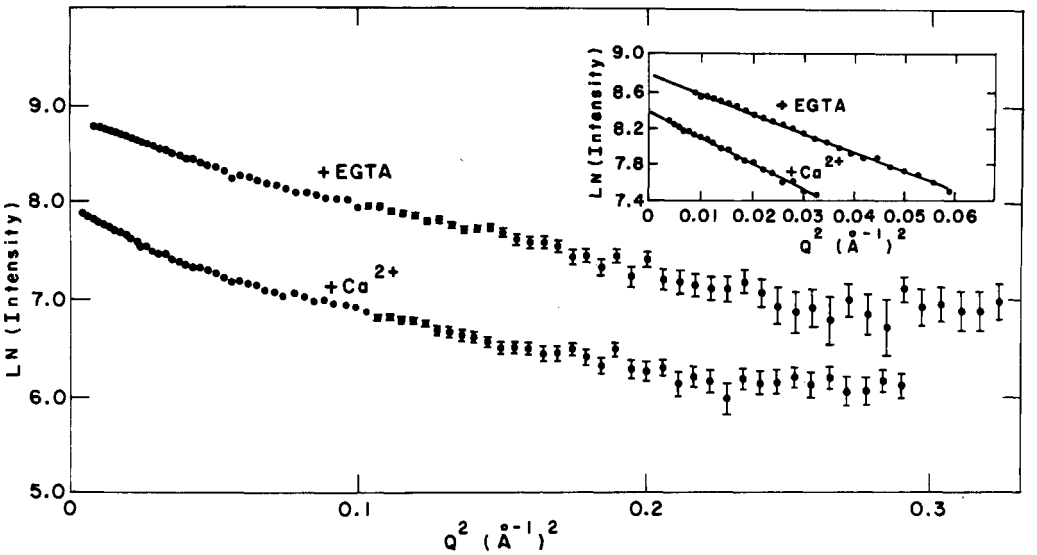

FIGURE 1: Typical extended scattering curves (inset: Guinier regions) for calmodulin in the presence of calcium or EGTA. For clarity, the values for calmodulin in the presence of calcium have been shifted by −1 unit on the ln I axis for the extended curve and by −0.4 unit for the inset, Guinier region.

100 mM KCl, and 50 mM MOPS–KOH buffer, pH 7.4 at 4 °C, changing the dialysis buffer every 24 h for 4–5 days. Atomic absorption measurements were made with an Instrumental Labs spectrometer and showed that <0.1 mol of Ca^{2+}/mol of calmodulin remained after this treatment.

Final dialysis fluids from all samples were retained for use in preparing serial dilutions and for measuring scattering of buffer alone.

Calmodulin concentrations were determined by the method of Lowry et al. (1951), calibrated by using a calmodulin solution of known concentration, determined previously by quantitative amino acid analysis.

Hen egg-white lysozyme samples were prepared by dialyzing protein against 150 mM NaCl and 40 mM sodium acetate, pH 3.8. Under these conditions, lysozyme is known to be monodisperse (Krigbaum & Kugler, 1970; Pickover & Engelman, 1982). The dialysis fluid was retained as before. The protein concentration was determined spectrophotometrically using $E_{282,1mg/mL} = 2.64$ after dialysis.

For the scattering experiments, up to eight different concentrations of each protein were prepared by gravimetrically diluting each dialyzed stock protein solution with its final dialysis fluid.

Irradiation of calmodulin for periods up to 18 h produced no change in the electrophoretic behavior of the protein on SDS gels or in calcium-dependent mobility changes on alkaline gels (Amphlett et al., 1976). The irradiated protein was also able to activate bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase in a manner quantitatively indistinguishable from the nonirradiated protein, using the assay procedure described previously (Birnbaum & Head, 1983).

X-ray Scattering Measurements. Data were collected on a small-angle X-ray scattering station at Yale by using the line source from an Elliott GX21 rotating anode generator, typically operated at 50 kV and 200 mA. Nickel-filtered X-rays from a copper anode were focused, by reflection from a glass mirror, at the plane of a position-sensitive detector. The sample was placed in a temperature-controlled holder located behind guard slits halfway between the mirror and the detector. The sample to detector distance was 56.2 cm. Data were recorded on a Canberra multichannel analyzer. The sensitivity of the position-sensitive detector was checked for uniformity before and after every set of runs. Backscatter from a lead beamstop was used to monitor the transmitted beam intensity. Sample temperature was kept constant at 20 °C during data collection. Typical data collection times ranged from 2 to 12 h.

Data Analysis. Data were analyzed as previously described (Levinson et al., 1983), using both the Guinier approximation (Guinier, 1939) and the indirect transformation method of Moore (Moore, 1980) to incorporate slit desmearing. The X-ray wavelength of 1.54 Å was used. For the Guinier analysis, data to $Q = 0.179$ Å^{-1} were used for calcium–calmodulin and to $Q = 0.236$ Å^{-1} for EGTA–calmodulin (where $Q = 4\pi \sin \theta/\lambda$). For the Moore analysis, data to $Q = 0.506$ Å^{-1} were used for all preparations. Direct comparisons in measured values such as radii of gyration and forward scatter were made only by using samples for which data collection conditions were the same, i.e., same cell and instrumental geometry.

Results

Extended scattering curves for calmodulin in the presence of calcium or EGTA are shown in Figure 1. The curve for protein in the presence of Ca^{2+} displays slightly more curvature. Representative Guinier plots are shown in the inset to Figure 1. No evidence of an upward curvature in these plots was observed at small angles. This indicates that no concentration-dependent aggregation of the protein occurs within the concentration ranges used: 9.1–36.1 mg/mL for calcium–calmodulin and 8.7–37.1 mg/mL for EGTA–calmodulin.

Radii of gyration were obtained from slit-desmeared values of data sets. Their dependence on protein concentration is shown in Figure 2. The linear increase with decreasing protein concentration observed in both plots, which is expected from interparticle interference, again indicates that there is no change in the aggregation state of the protein over the concentration ranges used. On extrapolation to zero concentration, the radius of gyration (R_g) of calmodulin in the presence of calcium is found to be 21.49 ± 0.18 Å and in the presence of EGTA 20.57 ± 0.15 Å. When calculated by the Guinier approximation from the line-smeared data, smaller values were obtained ($R_g = 20.3$ Å in the presence of calcium and $R_g = 18.9$ Å in the absence of calcium). The calculated contribution of calcium alone to the R_g of the calcium–calmodulin complex is no more than 0.2%, whereas from the slit-desmeared data,

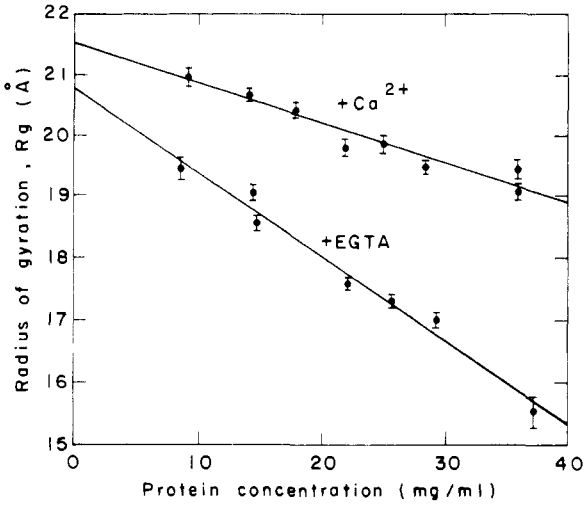

FIGURE 2: Typical protein concentration dependence of the apparent radii of gyration of calmodulin, from desmeared data used in the calculation of R_g values. The final R_g values at zero protein concentration derived by the combined analytical procedure (see text) are very slightly different from those seen in this figure.

a 4.5% difference in R_g between the calcium-bound and calcium-free forms of the protein is observed.

The differences exhibited by calmodulin in the presence or absence of calcium were reproduced by using three different preparations of protein and sets of scattering measurements. Further, calcium was dialyzed back into one sample that had been previously freed of calcium and for which X-ray scattering data had been measured. After calcium was added back, data were recollected and analyzed. The X-ray scattering characteristics for this sample were indistinguishable from those of calmodulin samples which had been prepared only in the presence of calcium.

The desmeared forward scatter, I_0, of an infinitely dilute solution is proportional to the molecular weight of the solute. The molecular weight of a protein therefore can be obtained by comparison of its forward scattering value with that of a protein of known molecular weight, assuming the same partial specific volume. The difference in partial specific volume is negligible for calmodulin in the presence of calcium or EGTA, 0.707 or 0.712 cm^3/g, respectively (Crouch & Klee, 1980); thus, they can be compared directly. Plots of I_0/c vs. c (data not shown), like those of R_g vs. c (shown in Figure 2), exhibit a linear increase with decreasing calmodulin concentration, again indicative of a constant aggregation state. I_0, when extrapolated to zero protein concentration, was found to be the same for calmodulin in the presence of calcium or EGTA. The molecular weight corresponding to this forward scatter value was determined as previously described (Levinson et al., 1983) using hen egg-white lysozyme as a standard (molecular weight 13 900; $\bar{\nu} = 0.71$). The molecular weight of calmodulin was found to be 16 500 ± 200, which agrees closely with the value of 16 680 derived from the amino acid sequence (Watterson et al., 1980).

The vector length distribution, or $P(r)$ function, which shows the number of interatomic vectors of a given length in the molecule, was derived from the scattering curve of calmodulin by the method of Moore (1980). In both the presence and absence of calcium, calmodulin appears to be an elongated molecule (Figure 3). For both forms of the protein, the maximum $P(r)$ value, which specifies the length of the greatest number of interatomic vectors in the molecule, falls near 18 Å. The shoulder corresponding to vectors having lengths of 35 Å and above must relate principally to distances between

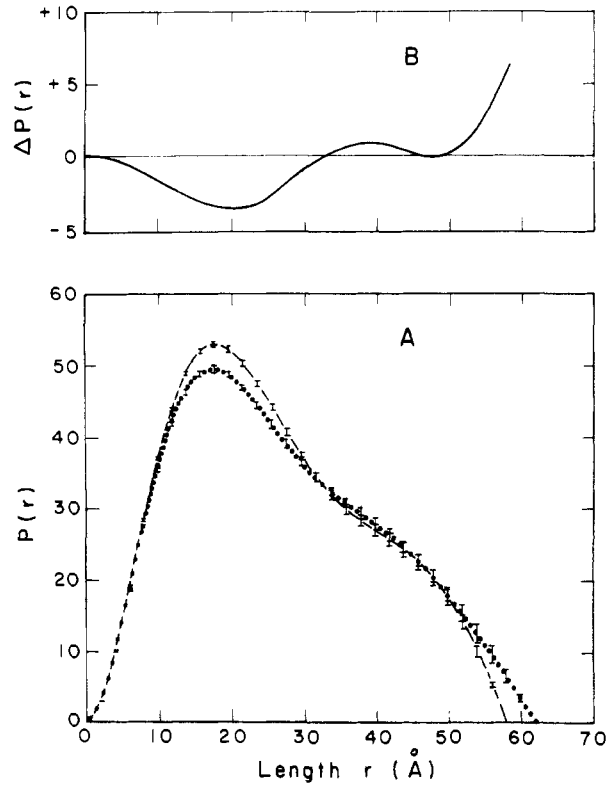

FIGURE 3: (A) Vector length distribution, $P(r)$, curve for calmodulin in the presence of calcium (···) or in the presence of EGTA (---). (B) Difference curve representing changes in the $P(r)$ function for calmodulin on binding calcium.

the two lobes of calmodulin and will represent interlobe vectors exclusively above 45 Å (see Discussion). The calcium-free form of calmodulin has a greater maximum $P(r)$ value, i.e., a larger number of vectors, at about 18 Å, and a smaller maximum vector length, by approximately 4 Å, than the calcium-containing form.

Discussion

The crystal structure of calmodulin shows a long central helix which connects the two halves of the molecule and forms the backbone of the protein. If this helix is flexible, the molecule in solution could have a preferred conformation different from that found in the crystal. The parameters determined by X-ray scattering can be compared to those of a number of models to assess whether the gross solution structure of calmodulin is consistent with that of the crystal structure. Assuming a molecular weight of 16 700 and a partial specific volume of 0.71 cm^3/g (Crouch & Klee, 1980), a spherical calmodulin molecule would have an R_g of 13.0 Å, considerably less than that observed for either the calcium or the EGTA form. A prolate ellipsoid is also an inadequate model. Assuming the same molecular volume and an R_g value of 21 Å, a prolate ellipsoid model of calmodulin would have an axial ratio near 4.35, which corresponds closely to that derived from the observed frictional coefficient (Crouch & Klee, 1980). However, such a prolate ellipsoid would have a length close to 90 Å, which is about 50% longer than the X-ray scattering and crystal structure data indicate. A "dumbbell" model based loosely on the crystal structure of calmodulin, consisting of two 20-Å-diameter spheres separated by a 24-Å-long rod with a diameter of 12 Å, has an R_g of 20.7 Å. The length and R_g for this model closely approximate the values determined for calmodulin in the present study. The $P(r)$ function for calmodulin is also consistent with the

dumbbell model, having a maximum number of vectors at about 18 Å, which will be dominated by vectors within each lobe, and a shoulder at 35 Å and vectors up to 62 ± 1 Å, which will principally represent distances between atoms in the different lobes. Vectors at greater than 45 Å will arise exclusively from lobe–lobe correlations since no other vectors of this length are possible. While our results are consistent with the dumbbell model, and generally fit the crystal structure, it is not possible to conclude that no other structure could fit these characteristics.

The effects on the scattering parameters of removing calcium from the calmodulin molecule are consistent with the dumbbell model in which there is a movement of the lobes toward one another. From the conformational differences between the calcium-filled and calcium-free cation binding domains observed in the troponin C crystal structure (Sundaralingam et al., 1985; Herzberg & James, 1985), it has been deduced that calcium binding induces a lengthening of the troponin C dumbbell structure (Sundaralingam & Rao, 1985). Similarly, it was suggested (Babu et al., 1985) that the two lobes of calmodulin may partially wrap around the long central helix in the absence of calcium, which would presumably also contract the overall protein structure. The results of the SAXS studies show that the radius of gyration of calmodulin is about 5% smaller for the protein in the absence of calcium than in its presence. The maximum vector length values derived from the data also show that calmodulin is shorter by about 4 Å in the calcium-free state than in the calcium-bound form. The corresponding increase in vectors at 18 Å could be associated with the incorporation of some of the central helix into the lobes, such as would be expected if lobes wrapped around the helix as suggested by Babu et al. (1985). With the dumbbell model, movement of the 20-Å spheres toward one another by a distance of 2.5 Å, close to that observed, would be sufficient to bring about the 1-Å change in R_g.

The magnitude of the observed R_g values (~21 Å) is comparable to Stokes radius values determined in sedimentation velocity studies (Crouch & Klee, 1980). However, the results of the sedimentation velocity studies suggest a decrease in the Stokes radius from 21.4 to 20.9 Å on binding calcium whereas the SAXS results show an increase in the R_g from 20.6 to 21.5 Å. The reason for the discrepancy in observations is unclear but may involve hydration effects, to which SAXS is less sensitive than hydrodynamic techniques such as sedimentation velocity. A decrease in the net charge associated with the binding of calcium to calmodulin might be expected to reduce the hydration shell and lead to a smaller Stokes radius, despite a conformational change leading to an increase in the size of the protein molecule itself. Such a change in charge was suggested to account for the 58% decrease in the second virial coefficient determined from the ultracentrifugation studies. We observe evidence of similar effects in the slopes of line-smeared and desmeared R_g vs. c plots. The slopes of these lines, which arise from interparticle interference effects, are proportional to the second virial coefficients. We find from our calmodulin data that the ratios of the slopes (calcium/EGTA) are also near 60%.

The observation from the X-ray scattering studies that bovine brain calmodulin is monomeric at physiological pH and ionic strength is in agreement with the results of sedimentation equilibrium ultracentrifugation studies (Crouch & Klee, 1980). From the SAXS study, it appears that calmodulin is monomeric at concentrations up to at least 37 mg/mL, whether or not calcium is present. In contrast, troponin C has been found, in sedimentation studies, to undergo a calcium-enhanced dimerization under similar conditions (Margossian & Stafford, 1982).

The results of the present study show that the calmodulin molecule, which exists in solution as a monomer, undergoes a conformational change on binding calcium that leads to an increase in the length of the molecule and an increase in its R_g. The parameters determined for calmodulin are consistent with the solution structure of the protein being the same as that of the crystal, i.e., a dumbbell structure. The results also support the hypothesis that the calcium-induced conformational change in calmodulin includes a movement of the two lobes of the molecule away from each other.

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