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Calmodulin and Troponin C Structures Studied by Fourier Transform Infrared Spectroscopy: Effects of Ca^{2+} and Mg^{2+} Binding†

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Received July 29, 1988; Revised Manuscript Received September 20, 1988

ABSTRACT: Fourier transform infrared (FTIR) spectroscopy has been used to examine the conformationally sensitive amide I′ bands of calmodulin and troponin C. These are observed to undergo a sequence of spectroscopic changes which reflect conformational rearrangements that take place when Ca^{2+} is bound. Calmodulin and troponin C show similar though not identical changes on Ca^{2+} binding, and the effect of Mg^{2+} on troponin C is quite different from that of Ca^{2+}. Both proteins show absorption maxima in the amide I′ region at 1644 cm^{-1} which is significantly lower in frequency than has been generally observed for proteins that contain a high percentage of α-helix. It is proposed that an unusually high proportion of the helices in the structures of these proteins are distorted from the normal α-helical configuration such that the carbonyl stretching frequencies are lowered. It is further proposed that the shift to lower frequency is due to backbone carbonyl groups in the distorted helices that form strong hydrogen bonds with solvent molecules. A decrease in intensity at 1654 cm^{-1}, the normal frequency assignment for α-helical structure, is observed as Ca^{2+} binds to calmodulin and troponin C. This suggests that Ca^{2+} binding results in a net decrease in "normal" α-helix conformation. There is a corresponding increase in intensity of the band at 1644 cm^{-1}, possibly due to an increase in distorted helix content, allowing for a net increase in helix consistent with circular dichroism estimates of the Ca^{2+}-dependent changes in helix content in calmodulin.

Calmodulin is a ubiquitous Ca^{2+} binding protein that regulates a wide variety of cellular functions including cyclic nucleotide phosphodiesterase (Dedman et al., 1977; Huang et al., 1981; Cox et al., 1981), adenylate cyclase (Valverde et al., 1979), phosphorylase kinase (Cohen, 1980), and myosin light chain kinase (Walsh et al., 1979; Bartelt et al., 1987). Troponin C is the Ca^{2+} binding protein involved in regulation of muscle contraction (Herzberg et al., 1986). Troponin C and calmodulin are thought to be evolutionarily related since they show a high degree of structural homology and can cross-react in their respective biochemical systems [see Means and Dedman (1980) and references cited therein]. They belong to a larger class of structurally related low molecular weight Ca^{2+} binding proteins that also includes parvalbumin.

Both calmodulin and troponin C bind up to four Ca^{2+} ions. In calmodulin, Ca^{2+} binding results in an increase in radius of gyration and a lengthening of the molecule (Seaton et al., 1985; Heidorn & Trewhella, 1988) which is accompanied by an increase in α-helix content, as estimated by a variety of spectrscopic techniques. These changes result in the exposure of hydrophobic regions of the molecule that are thought to be important in interactions with target enzymes (Burger et al., 1984). The conformational changes induced by Ca^{2+} binding to troponin C result in a shift in the position of tropomyosin, allowing contact between myosin and the actin thin filament.

The crystal structures of both calmodulin (Babu et al., 1985) and troponin C (Herzberg & James, 1985; Sundaralingam et al., 1985; Satyshur et al., 1988) have been solved. In both cases, the proteins were crystallized in the presence of excess Ca^{2+} and at low pH (approximately 5.5). The calmodulin structure was determined with all four Ca^{2+} sites occupied, while in the case of troponin C only two of the four sites were occupied. Both proteins showed unusual "dumbbell" structures consisting of two globular domains at opposite ends of an interconnecting α-helical segment which is largely exposed to solvent and makes few contacts with the rest of the molecule. The globular domains contain two Ca^{2+} binding sites each and show a high degree of structural homology with each other and with parvalbumin. The Ca^{2+} binding sites have a helix-loop-helix structural motif which is widely conserved among

† This work was supported by NIH Grant GM40528-01 and by Institutional Support for Research and Development Grant X81M (Los Alamos National Laboratory).
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0006-2960/89/0428-1294$01.50/0

Ca^{2+} binding proteins (Kretsinger & Barry, 1975).

The Ca^{2+} binding domains of both troponin C and calmodulin are designated I, II, III, and IV in order of their position in the sequence from the amino-terminal end. Troponin C has two high-affinity (III and IV) and two low-affinity (I and II) Ca^{2+} binding sites (Potter & Gergley, 1975). The two low-affinity sites are specific for Ca^{2+}, while the two high-affinity sites can alternatively bind Mg^{2+}. The calmodulin Ca^{2+} binding sites also show varying affinities, and the order of binding is II–I–III–IV, but the difference in binding affinities between high- and low-affinity sites in calmodulin is much smaller than in troponin C (Haiech et al., 1981).

The structure and conformation of calmodulin and troponin C have been extensively examined using a variety of spectroscopic techniques. Circular dichroism (CD) studies (Hennessey et al., 1987) of calmodulin show a small increase (4%) in α-helix content when 4 mol of Ca^{2+} is bound and a larger increase in helix content with increasing ionic strength. The authors of this study concluded that the Ca^{2+}-dependent structural rearrangement is so small that Ca^{2+}-dependent activation by calmodulin must be achieved by a structural reorientation of calmodulin with respect to the target enzyme rather than any internal conformational rearrangement. ^1H NMR data show Ca^{2+}-dependent changes in resonances assigned to residues located in the interconnecting helix region as well as in both globular domains, indicating widespread Ca^{2+}-dependent conformational changes in calmodulin (Klevit et al., 1985). Far-UV CD data also indicate the molecular structure of calmodulin varies with the addition of each Ca^{2+}. Binding the first Ca^{2+} leads to half the maximum increase in α-helix structure, while the maximum value is reached only when the third Ca^{2+} binds (Burger et al., 1984). Titration of calmodulin with the hydrophobic fluorescent probe 6-(4-toluidino)-2-naphthalenesulfonic acid (TNS) shows that the binding of two Ca^{2+} ions is sufficient to allow the interaction of TNS with calmodulin (Burger et al., 1984). Optical rotary dispersion has provided complementary estimates of the change in secondary structure of calmodulin on Ca^{2+} binding, also suggesting an increase in α-helical structure (Liu & Cheung, 1976).

Infrared spectroscopy is becoming an increasingly useful tool for examining protein conformation because of the increase in sensitivity and data acquisition rates that came with the development of Fourier transform methods for data acquisition and analysis. The technique capitalizes on the sensitivity of the stretching vibrations of peptide groups to their structural environment. Of particular interest in the case of proteins in D_2O solutions is the slightly deuterium-shifted amide I band, amide I′, which occurs between 1620 and 1700 cm^{-1} and is due primarily to the carbonyl stretching vibrations contributed by each peptide linkage in the protein. (D_2O is the preferred solvent for infrared studies of proteins because of strong absorbance by H_2O in the region of interest.) On the basis of a survey of the FTIR spectra of 21 proteins of known structures, Byler and Susi (1986) have assigned 11 well-defined frequencies in the amide I′ region to secondary structure elements. Using the measured areas of these components, they were able to estimate the secondary compositions for each protein, and these values are in good agreement with the X-ray data of Levitt and Greer (1977).

Because of the unusual nature of the calmodulin and troponin C structures, we thought it would be useful to examine the effects of Ca^{2+} binding on these proteins using FTIR spectroscopy. The effects of Mg^{2+} on the structure of troponin C were also examined. This was of particular interest because of previous small-angle X-ray scattering studies (Heidorn & Trewhella, 1988) that compared the solution structure of troponin C with Mg^{2+} to the crystal structure of troponin C with the two high-affinity Ca^{2+} binding sites occupied only by Ca^{2+}. FTIR provides structural information similar to that derived from CD, and both techniques rely on the consensus information built up from the examination of proteins with known structures. However, the infrared experiments are complementary to CD because they involve sampling a totally different physical phenomenon. In addition, better resolution of the component bands arising from the different secondary structure elements can potentially be achieved in FTIR spectra.

Materials and Methods

Sample Preparation. Bovine brain calmodulin was obtained from Calbiochem and assayed as better than 99.8% pure by gel electrophoresis. Highly purified turkey troponin C was isolated in Michael James Laboratory by Natalie Strynadka. Samples were dialyzed extensively against 5 mM EGTA in 50 mM MOPS (pH 7.4) and 100 mM KCl to assure complete removal of any residual Ca^{2+} from the protein. EGTA, which has carbonyl stretching bands that absorb strongly in the amide I′ region, was removed by dialyzing against MOPS–KCl for 48 h followed by 48 h against H_2O. The dialyzate was changed every 8 h. All samples were lyophilized and exchanged in D_2O (Sigma 99.96%-D) 3 times, allowing 4 h at 5 °C for thorough exchange. The final D_2O-exchanged samples were brought up in the standard buffer conditions: 50 mM MOPS (pD 7.0), 100 mM KCl. All reagents used had been D_2O exchanged 3 times. Protein concentrations were determined by amino acid analysis as described by Cohen et al. (1984) except acid hydrolysis of the protein samples was carried out for 48 h to ensure complete hydrolysis. All dilutions were done gravimetrically. The protein concentrations for the FTIR measurements shown were 1.45 mM for calmodulin and 0.56 mM for troponin C.

Ca^{2+} and Mg^{2+} Determinations. All solutions were analyzed to determine metal ion concentrations, and in particular Ca^{2+} and Mg^{2+} levels, before and after completion of the experiments. These analyses were carried out at the Colorado State University Soil Testing Laboratory using inductively coupled atomic emission spectrometry (Jarrell-Ash Model 975 ICP AtomComp spectrometer).

Infrared Spectroscopy. Infrared spectra were measured at 2 cm^{-1} resolution. Initial spectra were collected by using a Perkin-Elmer solution cell with Irtran windows and a 0.05-mm Teflon spacer. These windows were chosen to avoid potential contamination of the sample by dissolution of Ca^{2+} from, e.g., CaF_2 windows. However, the refractive index of the available Irtran windows was sufficiently different for the solutions measured, that low-amplitude, high-frequency ripples were observed in the spectra due to the spacing of the windows. The Ca^{2+} titrations were therefore repeated using CaF_2 windows, and the observed spectral changes due to Ca^{2+} addition were estimated to be the same as observed with the Irtran windows. No rippling was observed with the CaF_2 windows since they provided a much better match with the refractive index of the solutions. It was therefore concluded that, at least on the time scale of the FTIR measurements, insignificant amounts of Ca^{2+} could have been dissolved from the CaF_2 windows. This conclusion was later confirmed by the Ca^{2+} determinations that were done before *and* after FTIR measurements. All the spectra shown in this paper were, therefore, measured with the CaF_2 windows. To improve the signal to noise ratio, 1024 scans were co-added and triangularly apodized using a Mattson (Alpha Centauri) FTIR spectrometer. To minimize inter-

ference in the region of interest by the rotational fine structure bands from trace amounts of water vapor, the optic bench was maintained under constant positive dry-N_2 pressure. Absorbance spectra were collected after a 20-min N_2 purge and ratioed against a single-beam background spectrum of the cell with no spacer, also collected after a 20-min N_2 purge. Spectra of buffer blanks were collected for each different buffer condition in the same way and subtracted from the sample spectra to give the infrared absorption spectra of the protein of interest free from any buffer or solvent contributions. Each titration series was repeated 2–3 times to ensure reproducibility of the results.

Data Analysis. Because FTIR spectra are composed of overlapping bands that have inherently large half-bandwidths (Mantsch et al., 1986), spectral analysis requires the use of resolution enhancement methods such as Fourier self-deconvolution, curve fitting, difference spectroscopy, and second-derivative analysis in order to assign frequencies and relative intensities to each of the component bands. For these analyses, the buffer-subtracted absorption spectra were converted from binary to ASCII files and transferred to a DEC VAX-750 for analysis.

Fourier deconvolution spectra were calculated according to the methods of Kauppinen et al. (1981). The absorbance spectrum was inverse Fourier transformed to give its interferogram which was multiplied by the reciprocal of the inverse Fourier transform of a Lorentzian function, $\exp(2\sigma\pi|x|)$, and the triangular squared apodization function $(1-|x|/L)^2$. The resulting interferogram was Fourier transformed to give a Fourier self-deconvolved spectrum. L is the truncation distance for the interferogram, and σ is the full width at half-height of the Lorenztian function. Choosing values for L and σ which are too large results in overdeconvolution of the spectra which can be recognized by the appearance of side lobe artifacts. The optimal value of σ corresponds to the narrowest peak in the deconvolved spectrum. The components of the deconvolved spectrum are fit using Gaussian curves, the intensities of which give the percent contribution of each component to the amide I′ band.

By taking the second derivative of a spectrum, subtle variations in line slope caused by contributions from multiple bands beneath the spectral envelope can be accentuated. Such spectral second derivatives (Lee et al., 1985; Cameron & Moffat, 1987) were calculated by using the Mattson software.

The protein concentrations and path length were kept as constant as possible for each titration series, but there were small variations of a few percent that could affect calculated differences between spectra. To correct for these variations, the buffer-subtracted spectra were normalized according to the total intensity in the amide I′ region (1620–1700 cm^{-1}).

Results

Figures 1 and 2 show the buffer-subtracted FTIR absorbance spectra for Ca^{2+}/Mg^{2+}-free calmodulin and troponin C with their respective difference spectra calculated from the Ca^{2+} titration data. Figure 2 also shows the difference spectra calculated for troponin C from the Mg^{2+} titration data. All spectra were normalized to the Ca^{2+}/Mg^{2+}-free spectrum, and the differences were calculated by subtracting the spectrum for each Ca^{2+} or Mg^{2+} concentration from the Ca^{2+}/Mg^{2+}-free spectrum. Thus, positive peaks in the difference spectra correspond to a loss of intensity in the raw spectra with increasing Ca^{2+} (or Mg^{2+}), and vice versa. The ratios of moles of Ca^{2+} or Mg^{2+} bound per mole of calmodulin or troponin C were calculated by using the Adair equation (Burger et al., 1984) and the dissociation constants given by Haiech et al.

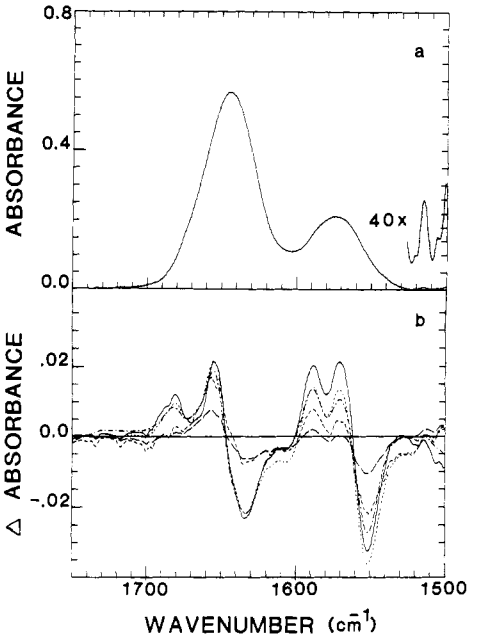

FIGURE 1: (a) FTIR absorbance spectrum of Ca^{2+}-free calmodulin in D_2O (100 mM KCl and 50 mM MOPS, pD 7.0); (b) Ca^{2+} titration difference spectra, calculated by subtracting the spectrum of each Ca^{2+}-bound form from the spectrum of the Ca^{2+}-free form. Moles of bound Ca^{2+} per mole of calmodulin for each difference are 3.99 (solid), 3.9 (dot), 3.18 (dash–dot), 2.09 (short dash), and 0.98 (long dash).

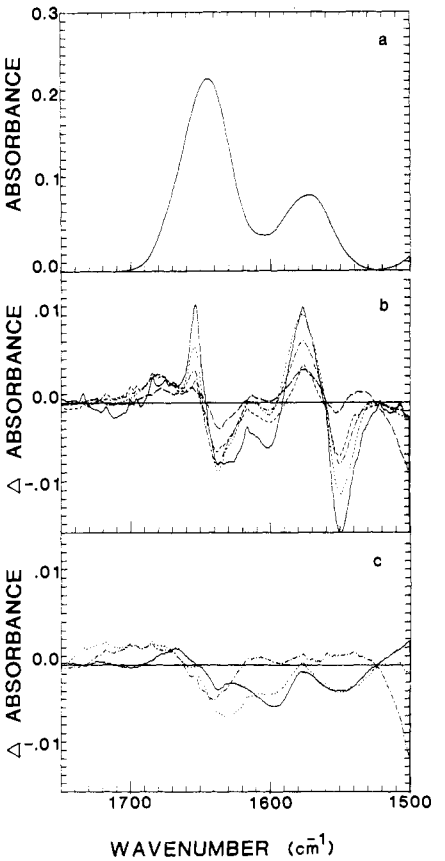

FIGURE 2: (a) FTIR absorbance spectrum of Ca^{2+}/Mg^{2+}-free troponin C in D_2O (100 mM KCl and 50 mM MOPS, pD 7.0); (b) Ca^{2+} titration difference spectra, calculated by subtracting the spectrum of each Ca^{2+}-bound form from the spectrum of the Ca^{2+}/Mg^{2+}-free form. Moles of bound Ca^{2+} per mole of troponin C for each difference are 3.99 (solid), 3.87 (dot), 2.82 (dash–dot), 1.98 (short dash), and 0.77 (long dash). (c) Mg^{2+} titration difference spectra calculated similarly to Ca^{2+} difference spectra in (b). Moles of bound Mg^{2+} per mole of troponin C are 1.83 (solid), 1.37 (dot), and 0.73 (dash–dot).

Table I: Relative Areas (as Percentages) of the Amide I′ Component Bands from the Fourier-Deconvolved Spectra of Calmodulin and Troponin C

mol of bound Ca^{2+}/mol of calmodulin	Calmodulin component frequencies (cm^{-1})					
	1628	1635	1644	1653	1663	1673
0.00	7.7	19.0	36.2	21.7	10.7	4.7
0.98	8.5	20.4	35.6	20.4	10.1	4.4
2.09	8.9	20.1	38.1	17.0	10.5	5.4
3.18	8.3	21.8	39.1	14.7	10.9	5.2
3.90	12.0	16.0	40.3	15.9	10.5	5.3
3.99	10.9	17.8	41.9	12.2	11.6	5.6

mol of bound Ca^{2+}/mol of troponin C	Troponin C component frequencies (cm^{-1})					
	1628	1636	1644	1652	1661	1674
0.00	6.7	22.7	26.9	23.1	14.2	6.5

(1981) for calmodulin and by Potter and Gergely (1975) for troponin C.

In the calmodulin spectra, the maximum in the amide I′ region occurs at 1644 cm^{-1}, under all conditions studied. In the amide I′ region of the difference spectra, increasing Ca^{2+} gives rise to a negative peak around 1634 cm^{-1} and positive features around 1655 and 1680 cm^{-1}. The changes at 1655 cm^{-1} are approximately 30% of the maximum on addition of the first Ca^{2+} and 80% of the maximum for the second. At 1534 cm^{-1}, the changes are 30% of the maximum for both the first and second Ca^{2+}, and maximal on addition of the third Ca^{2+}. These trends are reflected in the deconvolved spectra, though the enhanced resolution in these data allows for the assignment of specific component frequencies. The Fourier-deconvolved spectra for calmodulin consistently give six component bands in the amide I′ region (Figure 3a). These bands are also evident in the second-derivative spectra that are shown for comparison in Figure 3b. Relative intensities calculated for each component band by curve fitting the deconvolved spectra are given in Table I.

From the amide I′ assignments of Byler and Susi (1986), the 1628, 1635, and 1673 cm^{-1} components could be attributed to extended chain structure (low- and high-frequency components, respectively) and the 1663 cm^{-1} component to turns or bends. The 1673 cm^{-1} component could alternatively contain contributions from turns or bends. The remaining bands in the amide I′ region are at 1644 and 1654 cm^{-1}. At the highest Ca^{2+} concentration, the intensities of these bands are estimated at 42% and 12%, respectively. These frequencies correspond to the assignments given for unordered structure and α-helix, respectively, by Byler and Susi (1986). The most notable feature of the Fourier-deconvolved spectra is that increasing Ca^{2+} concentration results in a gain in intensity (approximately 5.5%) of the 1644 cm^{-1} band and a loss of intensity (approximately 10.8%) in the 1654 cm^{-1} band compared with Ca^{2+}-free calmodulin.

Spectral changes are also observed for calmodulin with increasing Ca^{2+} concentration in the region 1500–1620 cm^{-1}. This region contains contributions from the amide II components (arising predominantly from N–H in plane-bending and C–N stretching modes of the amide bond) which are not deuterium shifted. Also present are contributions from the stretching modes of particular amino acid side chains. There is a well-resolved peak at 1515 cm^{-1} (see insert in Figure 1a) that can be assigned to unionized tyrosine. This peak is not affected by Ca^{2+}, indicating that the structural environment of the residue(s) contributing to the band is not altered. Calmodulin contains two tyrosine residues (99 and 138) that

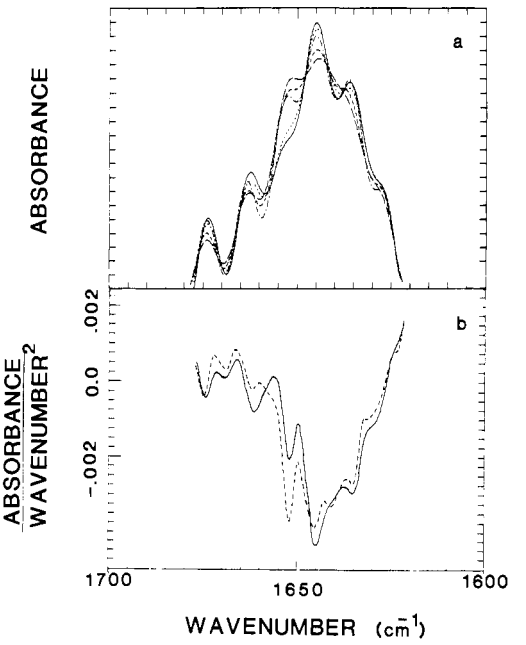

FIGURE 3: (a) Fourier-deconvolved amide I′ band of calmodulin with 3.99 (solid), 3.18 (dot), 2.09 (dash–dot), 0.98 (short dash), and 0 (long dash) mol of bound Ca^{2+}/mol of calmodulin; (b) second-derivative spectra for calmodulin without Ca^{2+} (dash) and with 3.99 mol of Ca^{2+} bound/mol of calmodulin (solid).

lie in Ca^{2+} binding domains III and IV, respectively. For troponin C, which contains no tyrosine, there is no peak at this frequency.

There are positive peaks in the difference spectra at 1570 and 1588 cm^{-1}. These peaks occur near the frequencies assigned to the carboxylate stretching modes of glutamic and aspartic acid side chains. Chirgadze et al. (1975) report the carboxylate stretching frequency of the functional group of aspartic acid at 1584 cm^{-1}, and of glutamic acid at 1566 cm^{-1}. The difference spectra also show a negative peak at 1550 cm^{-1} that varies inversely with the features at 1570 and 1588 cm^{-1}. This may arise from carboxylate stretching modes that have been shifted downfield due to Ca^{2+} binding to the carboxylate ligands in the ion binding pockets. Such interactions would weaken these carboxylate bonds, thus lowering their vibrational frequencies.

Finally, there is a negative feature in the difference spectra near 1605 cm^{-1}. This region is too low to be assigned to amide I′ contributions. Bands due to aromatic side-chain groups (Susi & Byler, 1983) or to the guanidine group of arginine (Chirgadze et al., 1975) have been reported in this region.

Like calmodulin, the maximum in the amide I′ band of troponin C occurs at 1644 cm^{-1}. There is also qualitative aggreement between the difference spectra calculated from the Ca^{2+} titration data for troponin C and calmodulin, though there are some differences. The changes in the amide I′ region are very similar in that the difference spectra show positive peaks at 1680 and 1654 cm^{-1} and a negative peak at 1637 cm^{-1}. However, the magnitude of the differences is larger in troponin C. For example, the difference at 1654 cm^{-1} for approximately 3.8 mol of bound Ca^{2+} per mole of protein is larger by approximately 20% when the spectra are normalized for concentration. Furthermore, the largest incremental change at 1654 cm^{-1} occurs for troponin C as the fourth Ca^{2+} site is bound, compared to calmodulin which shows the largest incremental change on binding at the first and second sites.

Similar to the calmodulin spectra, the Fourier-deconvolved spectra of troponin C show general agreement with the second-derivative spectra (Figure 4). The Ca^{2+}/Mg^{2+}-free

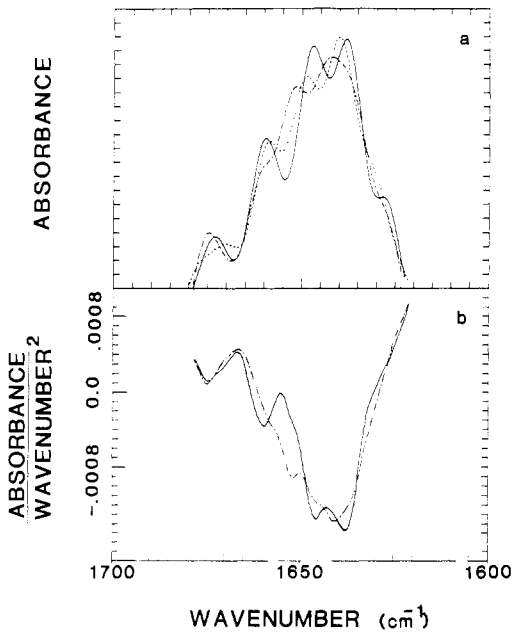

FIGURE 4: (a) Fourier-deconvolved amide I′ band of troponin C with 0.0 (dash–dot), 1.98 (dot), and 3.87 (solid) mol of bound Ca^{2+}/mol of troponin C; second-derivative spectra of troponin C without Ca^{2+} (or Mg^{2+}) (dash–dot) and with 3.87 mol of Ca^{2+}/mol of troponin C (solid).

spectrum of troponin C shows six component bands in the amide I′ region whose frequencies and relative intensities are very similar to the Ca^{2+}-free calmodulin spectrum (Table I). With increasing Ca^{2+} concentration, however, in addition to intensity variations in these component bands, there are shifts in frequencies (Figure 4a). This prohibits reliable estimates of these areas under different components as a function of Ca^{2+} concentration, though a general loss of intensity at 1654 cm^{-1}, and a gain in intensity at 1644 cm^{-1}, is evident in the deconvolved spectra.

In the region 1500–1620 cm^{-1}, the troponin C/Ca^{2+} difference spectra are again similar to those for calmodulin. The most notable difference is that there is a single broad peak at 1576 cm^{-1}, compared to the two peaks at 1570 and 1588 cm^{-1} in the calmodulin difference spectra. Some of the aspartic and glutamic acid residues that contribute in this region may be more strongly vibrationally coupled in troponin C than in calmodulin, since the peak at 1576 cm^{-1} occurs at an intermediate frequency between the peaks observed in the calmodulin difference spectra.

The effects of Mg^{2+} addition on the conformation of troponin C are quite different from those of Ca^{2+}. The Mg^{2+} changes induced in the spectra are smaller in magnitude than those caused by Ca^{2+}, and the difference spectra only show very broad features with no well-defined peaks. Furthermore, there is no evidence for any changes around 1654 cm^{-1}.

Discussion

The intensity maximum in the FTIR absorbance spectra of both calmodulin and troponin C in the amide I′ region is at 1644 cm^{-1}. This is a surprisingly low frequency for proteins considered to contain 50% or more α-helix. The intensities of the bands at 1644 and 1654 cm^{-1} calculated from the Fourier-deconvolved spectrum of calmodulin with four Ca^{2+} bound are 42% and 12%, respectively. Byler and Susi (1986) surveyed the FTIR spectra of 21 water-soluble globular proteins and consequently assigned the 1654 cm^{-1} frequency to α-helix structure and the 1644 cm^{-1} frequency to unordered structure. The bands assigned to α-helix and unordered structure in the Fourier-deconvolved spectra of the proteins surveyed deviated from these mean values by only ± 2 cm^{-1}, and the intensities calculated for each component band agreed with estimates of the secondary components from X-ray crystal structures within 3%, for most proteins. The total helix content of calmodulin, based on the crystal structure, is approximately 60%. From our own CD measurements (Kowluru et al., 1988), we estimate the helix content of Ca^{2+}-saturated calmodulin (100 mM KCl and 50 mM MOPS, pH 7.4) to be 53%, though other investigators find varying numbers for this estimate [e.g., Hennessey et al. (1987) quote a value of 61% which is much closer to the crystal structure estimate]. The sum of the 1654 and 1645 cm^{-1} band intensities gives a value of 54% which is very close to our CD estimates. This raises the question as to whether the 1654 and 1644 cm^{-1} bands arise, respectively, from "normal" α-helix structure, as observed in the globular protein structures of Byler and Susi's basis set, and some sort of distorted α-helix structure that results in a shift to lower frequency of its amide I′ band. It is also possible that the 1644 cm^{-1} band is a combination of contributions from unordered and distorted helix structures.

Unusually high frequencies for α-helical structure (at 1664 cm^{-1}) have been reported for bacteriorhodopsin by Rothschild and Clark (1979). This shift to higher frequency was explained by Krimm and Dwivedi (1982) as the result of weaker hydrogen bonding in the α_{II}-helix they propose for bacteriorhodopsin, and a consequent increase in the amide I carbonyl stretching force constant. Parrish and Blout (1972) published spectroscopic data on solutions of the helix forming poly(L-alanine). FTIR spectra of poly(L-alanine) in hexafluoroisopropyl alcohol (HFIP) showed an amide I band that was shifted 5–10 cm^{-1} down from the normal α-helix band assignment. When the HFIP was evaporated away to leave a poly(L-alanine) film, the usual α-helix spectrum was obtained. Parrish and Blout concluded that the observed shift to lower frequency arose from the fact that in the strong hydrogen bond forming solvent HFIP, the helical conformation adopted by poly(L-alanine) was not the classical α-helix. They proposed a "doubly hydrogen-bonded helix" in which the peptide carbonyls point slightly out from the helix axis and are simultaneously hydrogen bonded to the NH of the fourth peptide residue on the carbonyl terminal side (as in a classical α-helix) and to a solvent molecule's hydroxyl hydrogen. A bond arrangement of this type would result in a weakening of the carbonyl bond and, consequently, a lowering of the amide I carbonyl stretching frequency. They further point out that a helix with ϕ, ψ angles near $-65°$, $-40°$ would facilitate the formation of two hydrogen bonds with each carbonyl group.

In their description of the high-resolution structure of chicken skeletal muscle troponin C, Satyshur and colleagues (Satyshur et al., 1988) describe a large number of solvent interactions with backbone carbonyls. In particlar, there are 68 well-defined water molecules that solvate the α-helices, loops, and linkers. Twelve backbone carbonyls in the middle of helical regions are found to form hydrogen bonds with water molecules, and 20 water molecules appear to form strong interactions with the carbonyl oxygens of the metal binding sites and linker regions, and also form intramolecular bridges between carbonyl groups at the C-termini of helices. In the center of the interconnecting helix, a unique configuration is observed in which water molecules are bonded to backbone carbonyl groups of residues 86, 87, 88, and 90, girdling almost a full turn of helix. In helix B, water molecules are observed to penetrate and interpose themselves between the backbone carbonyl and amide groups between several residues. The Ramachandran plot calculated from the ϕ, ψ angles of the

refined structure shows a distribution that is strongly skewed from the canonical Pauling–Corey α-helix values of −48°, −57° (Pauling et al., 1951) toward the values of −65°, −40° that would favor doubly hydrogen-bonded helix formation.

Double hydrogen bonding in α-helices has been observed in other proteins. Blundell and colleagues (Blundell et al., 1983) considered solvent-induced distortions and curvature in α-helices in globular proteins and found that the Pauling–Corey description of α-helices was not a good model for helices in proteins based on crystallographic data. In fact, they found the mean values of ϕ and ψ for helices in proteins are usually closer to −63° and −42°. They cite examples of peptide groups in helices with carbonyl groups rotated to form a strong hydrogen bond with solvent molecules and a hydrogen bond with the fourth next peptide amide that is quite nonlinear. The examples of this double hydrogen bonding that have been observed in other globular proteins usually involve one or a few interactions on one side of a helix that result in a kink in the helix. More recently, Barlow and Thornton (1988) surveyed helix geometries in 57 known protein structures including 16 structures determined to high resolution (but not including troponin C). They found mean values for ϕ and ψ angles in α-helices of −62° and −41°.

On the basis of the FTIR studies of poly(L-alanine) in HFIP, the crystal structure of calmodulin, and the refined crystal structure of troponin C that describes a high degree of solvent penetration in the structure, we propose that the intensity maximum at 1644 cm^{-1} in the FTIR spectra of calmodulin and troponin C is due to an increased proportion of distorted helix structure in which the backbone carbonyls form strong hydrogen bonds with solvent molecules or side chains. While other globular proteins show helix distortions and double bonding of the backbone carbonyls to solvent and side-chain hydroxyls, we propose that there is a relative increase in these types of solvent interactions in calmodulin and troponin C. We further suggest that this arises from the dumbbell-shaped structure of these proteins that provides a highly exposed central interconnecting helix region for solvent interactions and also results in a higher ratio of surface area to volume compared with other, usually more compact, globular proteins. (The surface area to volume ratio for a dumbbell with similar dimensions to calmodulin is 30% greater than the surface to volume ratio of a sphere with the same volume.) Satyshur and colleagues (Satyshur et al., 1988) also describe some bifurcated hydrogen bonds in helices A and C in which the backbone carbonyl group forms relatively weak hydrogen bonds to both the third and fourth residues' amide hydrogen. It is not clear, however, whether these interactions would lead to a strengthening or weakening of the carbonyl bond. Resolution of this question would require quantitation of the combined strength of the two nonideal hydrogen bonds relative to a normal hydrogen bond.

The alternative explanation for the amide I′ maxima occurring at 1644 cm^{-1} is that there is much more unordered structure in calmodulin and troponin C in solution compared with the crystal structures. While small-angle scattering data suggest some conformational differences between the crystal and solution forms, the evidence also suggests that the structure of the globular domains is largely conserved and the changes are confined mostly to the interconnecting helix region (Heidorn & Trewhella, 1988). The data do not support a large-scale loss of ordered structure that would account for the 1644 cm^{-1} maximum.

Because the conformationally sensitive amide I′ bands overlap, several complementary methods of data analysis have been used in these studies. Second-derivative analysis, Fourier self-deconvolution, and difference spectroscopy have each given qualitatively consistent results. Some variations are observed in the quantitation of differences using spectral subtraction and curve fitting of Fourier-deconvolved spectra. These differences reflect the fundamental limitations of resolving multiple bands in the spectra. It is important to note here that the intensity values quoted under Results (Table I) are determined by fitting the deconvolved spectra with multiple bands whose amplitudes, frequencies, and full widths at half-maximum are allowed to vary. The uncertainty in the percentage areas under the component bands is greatest when the fitted bands are not well resolved, as is the case for calmodulin and troponin C. The difference spectra are expected to give more reliable estimates of intensity variations, but they are limited by peak overlap; therefore, it is uncertain how much each peak is contributing to a given difference. We have therefore avoided interpreting the observed variations in intensities too quantitatively but rather have given weight to trends that are consistent between the different approaches to analysis. However, the difference between the estimated helical content from crystallography and the intensity of the 1654 cm^{-1} band is significant and cannot be accounted for by uncertainty in the fitting procedures.

The FTIR difference spectra reveal the sequence of conformational changes which take place when calmodulin and troponin C are titrated with Ca^{2+}. The most striking and consistent result of the Ca^{2+} titrations for calmodulin and troponin C is that increasing Ca^{2+} results in a loss of intensity at 1654 cm^{-1}, the frequency usually assigned to α-helix conformation in globular proteins. From the Fourier-deconvolved calmodulin spectra, this loss of intensity is estimated at approximately 10%. There is a corresponding increase in intensity of approximately 6% at 1644 cm^{-1}. While it was not possible to quantitate these changes in troponin C, the trends are similar to what is observed for calmodulin as determined from the difference spectra. Because the band at 1644 cm^{-1} is likely to contain contributions from both unordered structure and helix distorted through solvent and side-chain interactions, it is not possible simply to add the differences to estimate the total change in helix content on Ca^{2+} binding for calmodulin. Previous spectroscopic data, in particular from CD studies, support the fact that Ca^{2+} binding causes an increase in total helix content. For there to be consistency between the CD and FTIR data, it appears that Ca^{2+} binding causes a loss of "normal" α-helix conformation that is accompanied by an increase in the distorted helix conformation and a decrease in unordered structure. The FTIR data are most clear in this respect for calmodulin. Troponin C is similar to calmodulin, but the small shifts in frequencies for the amide I′ component bands indicate this structure undergoes some additional conformational rearrangement on Ca^{2+} binding.

It might be speculated that the proposed increase in the distorted helix structure on addition of Ca^{2+} is connected to the observation that Ca^{2+} binding results in an expansion of the calmodulin structure, hence further increasing the potential for the formation of solvent interactions that result in helix distortions. An increase in these interactions may provide a mechanism for flexing of the interconnecting helix, which could play a role in triggering Ca^{2+}-induced activation by calmodulin and troponin C. Satyshur and colleages (Satyshur et al., 1988) have already suggested that interactions between water molecules and the backbone of troponin C are a possible mechanism for flexing of the interconnecting helix and hence altering the relative dispositions of the globular domains of

troponin C to optimize the interaction with troponin I that would trigger muscle contraction. A similar mechanism could be invoked for Ca^{2+}-induced activation of target enzymes by calmodulin. In the calmodulin case, flexing in the interconnecting helix could result in optimizing the disposition of target enzyme binding sites on the two globular domains of calmodulin.

Ca^{2+} and Mg^{2+} clearly have quite different effects on troponin C structure as estimated by the spectral changes both in the amide I′ region as well as in the region 1500–1620 cm^{-1}. In particular, there are no observed changes at 1654 cm^{-1} due to Mg^{2+} that characterize the Ca^{2+} effects. In earlier small-angle X-ray scattering studies (Heidorn & Trewhella, 1988), it was concluded that the solution structures of calmodulin and troponin C differed from the crystal structures in that the globular domains of both proteins were closer together, on average, in solution. This required some rearrangement of the structure in the interconnecting helix region. In the case of calmodulin, the Ca^{2+}-saturated crystal and solution forms were compared, and good agreement was obtained between the solution data and a model that was derived from the crystal structure by placing a "bend" in the density between the two globular domains. In the case of troponin C, Ca^{2+}-induced protein aggregation and the scattering data were therefore difficult to interpret. However, Mg^{2+} did not induce aggregation, and since the crystal structure had only the two high-affinity sites occupied by Ca^{2+}, and it is believed that Mg^{2+} binds only to the two high-affinity sites, the Mg^{2+}-bound solution form was used to compare with the crystal form. This comparison showed differences between the solution scattering and crystal structure data that could not be accounted for by structural changes in only the interconnecting helix region. Specifically, there was a discrepancy in the vector distribution functions, $P(r)$, for vectors between 10 and 15 Å. These vectors arise from points within one globular domain. The discrepancies could be accounted for by modifying the structure of the globular domains to move density from the globular domains into the interconnecting region. The FTIR data suggest that there are differences in the globular domain structure of troponin C with Mg^{2+} bound compared with Ca^{2+} bound that might give rise to the discrepancies in the $P(r)$ functions.

Independent estimates of the secondary structure of calmodulin from CD analysis have varied considerably, and *the FTIR data suggest one cannot interpret spectroscopic data from these proteins in terms of "normal" or "average" globular protein structures*. Secondary structure estimates based on a reference set of proteins of known structure are subject to considerable uncertainty for both calmodulin and troponin C because their secondary structures differ in important ways to those of the reference set. In spite of these limitations, this application of FTIR difference spectroscopy to the problem of metal complexation in Ca^{2+} binding proteins has proven to be extremely sensitive to subtle conformational and molecular transitions.

ACKNOWLEDGMENTS

We thank Michael N. James for helpful discussions and for providing the resources for the isolation and purification of the troponin C used in these studies. We also greatly appreciate discussions with Robert Woody that led to the model to account for the shifted amide I′ contributions, and with Thomas Whaley on hydrogen-bonding schemes. We thank John Rodkey for providing programs to translate the FTIR data into the ASCII format. We thank Nancy Downer for useful discussions on the application of FTIR to calmodulin structure analysis. We thank Rex Hjelm for critically reading the manuscript.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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Rapid Kinetic Analysis of the Calcium-Release Channels of Skeletal Muscle Sarcoplasmic Reticulum: The Effect of Inhibitors

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ABSTRACT: During excitation of skeletal muscle fibers, Ca ions stored in the cisternal compartments of the sarcoplasmic reticulum (SR) are released to the cytosol within milliseconds. In this study, the kinetics of the fast release of Ca were analyzed by means of a newly developed rapid filtration apparatus. Isolated SR vesicles of cisternal origin were preloaded with 1 mM $^{45}CaCl_2$, and Ca efflux was studied (between 20 and 1000 ms) after dilution into media of various composition. The effect of extravesicular Ca on the gating of the Ca-release channels and its susceptibility to the influence of drugs were thoroughly investigated. In the presence of 1 mM $MgCl_2$ and 3 mM ATP, highest rates of Ca release were observed at a free Ca concentration between 1 and 50 μM. In the lower micromolar Ca range, compounds such as neomycin and FLA 365 inhibited the release monophasically and with an IC_{50} of 0.37 and 3.4 μM, respectively. At Ca concentrations between 10 and 50 μM, the inhibitors could not block Ca release effectively. Close analysis of the dose–response curves revealed a biphasic pattern, indicative of the presence of two substates of the Ca-release channel, displaying high- and low-affinity binding sites for the inhibitors. Interestingly, neomycin (or ruthenium red) and FLA 365 at low concentrations acted synergistically and blocked release completely. The results indicate the existence of various open substates of the Ca channels that can be distinguished pharmacologically. Effective blockade of rapid Ca release requires inhibition of all substates coexisting under a given condition.

Rapid Ca uptake and release from the sarcoplasmic reticulum (SR) play a key role in the regulation of the kinetics of relaxation and contraction of the myofilaments in striated muscle (Endo, 1977). The basic mechanism of Ca translocation into the SR compartment, which is effected by a Ca-pumping ATPase, is well characterized [for a review see, e.g., Inesi (1985) or Ikemoto (1982)]. On the other hand, the coupling of the excitation wave spreading along the plasma membrane and into the transverse tubular system with massive release of Ca ions from the adjacent terminal cisternae of the SR is not yet fully understood [for a review see Martonosi (1984)]. In-depth characterization of the phenomenon has long been impeded by its morphological and organizational complexity as well as the high rates of Ca release, for the investigation of which special rapid kinetic methods are required. Only very recently have consistent advances in our knowledge of the molecular organization of the Ca-release process been achieved, thanks to the availability in radiolabeled form of ryanodine, a plant alkaloid capable of binding with nanomolar affinity to the cisternal compartments of the SR (Fleischer et al., 1985; Pessah et al., 1987) and of interfering with the operation of the Ca channels (Jones et al., 1979; Alderson & Feher, 1987). Solubilized ryanodine receptors have been purified by immunoaffinity chromatography (Imagawa et al., 1987), by centrifugation through a linear sucrose gradient (Lai et al., 1988), or by differential affinity chromatography (Inui et al., 1987) procedures. The identity of receptor and Ca-release channels has been proved by conductance-recording studies of the purified ryanodine receptor reconstituted in planar lipid bilayers (Imagawa et al., 1987; Lai et al., 1988). These investigations shed light on the electrical properties of the single Ca channel and provided useful information on the Ca-, Mg-, and nucleotide-dependent regulation of its activity. The influence of the latter compounds and of other agents, such as calmodulin and caffeine, on the activity of the Ca channels was also successfully investigated by using native SR vesicles with the help of rapid kinetic procedures (Nagasaki & Kasai, 1983; Meissner et al., 1984; Kim et al., 1983; Moutin & Dupont, 1988). In the present study, we used a recently developed rapid-filtration apparatus (Dupont & Moutin, 1987) to study the effect of some inhibitors of the Ca-release process in skeletal muscle SR. The method can resolve Ca-release kinetics from passively loaded SR cisternae in the millisecond time range. Among the compounds investigated, ruthenium red (RR) and the antibiotic neomycin have previously been described as inhibitors of Ca release from skeletal SR (Palade, 1987). Another inhibitor tested in this study was the recently discovered compound FLA

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0006-2960/89/0428-1301$01.50/0