BRIEF COMMUNICATION

A LASER RAMAN SPECTROSCOPIC STUDY OF CA^{2+} BINDING TO TROPONIN C

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ABSTRACT Laser Raman spectroscopy has been used to detect structural changes in troponin C induced by Ca^{2+} binding. Addition of Ca^{2+} to the high affinity Ca^{2+} – Mg^{2+} sites produces perturbations in the amide III region of the spectrum indicative of increased α-helical content, and in regions of the spectrum corresponding to carboxylate, thiol, and phenol side chains. However, Ca^{2+} binding to the low affinity Ca^{2+}-specific sites is not detected by laser Raman spectral changes.

INTRODUCTION

Troponin C (TnC) is the Ca^{2+}-binding subunit of troponin which, together with tropomyosin, regulates the interaction between actin and myosin in vertebrate skeletal muscle. TnC binds four calcium ions: there are two sites with a higher affinity ($K_{app} = 2 \times 10^7 M^{-1}$) that also bind Mg^{2+} ($K_{app} = 5 \times 10^3 M^{-1}$), and two with a lower affinity ($K_{app} = 5 \times 10^6 M^{-1}$) that are calcium-specific (1). A model of TnC structure based on its sequence homology with carp parvalbumin, whose crystal structure is known (2), suggests that each of the four binding sites consists of a ten-residue loop rich in aspartic and glutamic acid residues, flanked on either side by α-helical segments. Calcium binding to the two high affinity Ca^{2+} -Mg^{2+} sites induces large increases in secondary (α-helical) and tertiary structure, as indicated by various spectroscopic techniques (3–9). Calcium binding to the low affinity calcium-specific sites, however, produces more subtle changes detectable with the use of fluorescence probes (5, 10) and by proton NMR (7, 8).

In this work we have employed laser Raman spectroscopy as another tool to probe Ca^{2+}-induced changes in TnC. The Raman spectrum of a protein shows a series of peaks characteristic of the vibrational modes of the various bonds in it. Both the peptide backbone

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and the amino acid side chains contribute to the spectrum, and the identification of the peaks has been based primarily on comparisons with amino acids, model peptides, and well-characterized proteins (e.g., see references 11–13). The data presented here show that Ca^{2+} binding to the Ca^{2+}-Mg^{2+} sites of TnC produces changes in several regions of the spectrum, interpretable as increases in secondary structure and as changes in tyrosine, cysteine, and carboxylate side chains, whereas binding to the lower affinity Ca^{2+}-specific sites does not produce appreciable spectral changes.

METHODS

Rabbit skeletal TnC was prepared by the method of Potter and Gergely (1). Purified TnC was lyophilized after dialysis vs. 5 mM EDTA followed by several changes of distilled, deionized water. For spectroscopic studies, weighed samples of TnC were taken up in a solution containing 0.1 M KCl and 25 mM Hepes buffer at pH 7.4 ± Ca^{2+}. Ten microliter samples were sealed in Kimax glass capillary tubes (i. d. = 1 mm) at [Tnc] = 1–5 mM. Samples were mounted perpendicularly to the scattering plane and maintained at 10°C.

The computer-controlled laser Raman spectrophotometer used in this study has been previously described (14). The instrument was programmed to scan the spectral region from 580 cm^{-1} to 1,780 cm^{-1} at a rate of 5 cm^{-1}/s, signal averaging over 25 scans, giving a spectral resolution < 1 cm^{-1}. Regions of interest in the spectra were scale-expanded and, when necessary, appropriate corrections were made to obtain a flat base line.

RESULTS AND DISCUSSION

The amide III region of the Raman spectrum (1,225–1,275 cm^{-1}) encompasses peaks characteristic of protein secondary structure. The spectrum of *apo* TnC (Fig. 1) contains two broad major peaks in this region, namely at 1,242 and 1,268 cm^{-1}, and two smaller peaks, at 1,253 and 1,260 cm^{-1}. The two higher frequency peaks are characteristic of α-helical structure, while those at 1,242 and 1,253 cm^{-1} represent disordered structures. Peaks below 1,240 cm^{-1}, normally indicating β-pleats, are absent from the spectrum suggesting only a minor contribution of β-structure to the protein, in agreement with other studies (2, 15). The stepwise addition of Ca^{2+} ions to TnC (Fig. 1) results in changes in the spectrum that are consistent with an increase in α-helical content (16–22) and that are essentially complete at 2 mol metal /mol protein. The changes include a sharpening of the 1,260-cm^{-1} peak and the appearance of two shoulders at 1,272 and 1,280 cm^{-1}. More difficult to interpret are the changes at the lower frequencies of the amide III region; namely the increase in the 1,253-cm^{-1} peak and the appearance of an intense peak at 1,236 cm^{-1} with a shoulder at 1,232 cm^{-1}. While peaks in the 1,235-cm^{-1} region are generally interpretable as β-pleated sheets, the circular dichroic (CD) data (15) and models based on the homologous protein parvalbumin (2) for TnC preclude extensive contributions from such structures. Thus it is more likely that the triplet of peaks centered at 1,236 cm^{-1} represents nonhydrogen-bonded disordered structure, as has been reported by Yu et al. (20) for denatured insulin and proinsulin. One cannot, however, rule out a contribution to this region from the high affinity calcium-binding loops themselves which probably exist as somewhat extended chains in the absence of Ca^{2+} and in considerably tighter reverse (β) turns in its presence (6, 15). It is noteworthy that no substantial spectral changes occur in the amide III region as the third and fourth Ca^{2+} ions are added to the protein. Addition of Mg^{2+} to TnC produces qualitatively

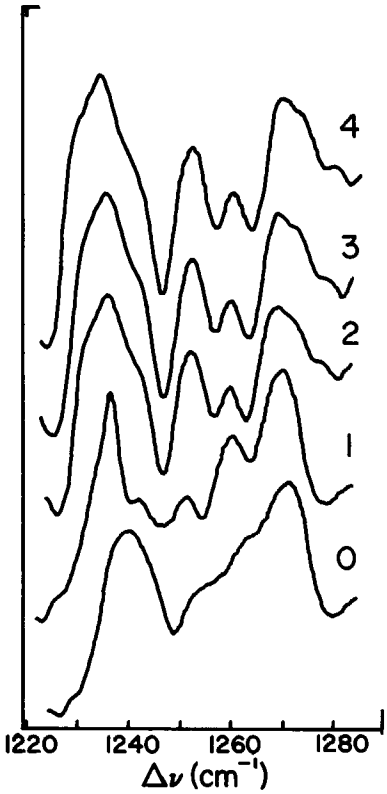

FIGURE 1 Expanded Raman spectrum of amide III region. Conditions: 1mM TnC in solution containing 0.1 M KCl, 25 mM Hepes buffer, pH 7.4, and Ca^{2+} at indicated mole ratios.

similar changes in the spectra of TnC (not shown). The low Mg^{2+} affinity for the protein, however, makes it difficult to obtain complete saturation of TnC with Mg^{2+}.

The fundamental conclusion to be drawn from the amide III region of the laser Raman spectrum is that major changes are associated with Ca^{2+} binding to the Ca^{2+}-Mg^{2+} sites rather than the Ca^{2+}-specific sites. van Eerd and Kawasaki (3) first showed large increases in the 222-nm ellipticity of TnC associated with higher affinity site Ca^{2+} binding. Their observations have been verified by several other studies (3–5, 15) which suggest an increase in the α-helical content from $\sim$ 35 to 50% upon the addition of Ca^{2+} to those sites. Studies on proteolytic fragments (6) further suggest that most of the increased secondary structure occurs in the C-terminal half of the molecule, in which the high affinity sites are located. These results are supported by proton NMR studies on both intact TnC (7–9) and proteolytic fragments.[1]

Structural changes associated with Ca^{2+} binding to the low affinity sites have generally been more difficult to delineate since the secondary and tertiary rearrangements that occur

[1]Evans, J. S., B. A. Levine, P. C. Leavis, J. Gergely, Z. Grabarek, and W. Drabikowski. Manuscript submitted for publication.

are apparently small. Several probes (5, 10) have shown changes upon saturating these sites, and proton NMR studies have indicated subtle tertiary structural changes (7–9, 22). CD spectra generally indicate very small (5–10%) secondary changes. Two recent studies (23, 24), however, show biphasic calcium titrations with up to 40% of the total CD change occurring when the two lower affinity sites are titrated. It is somewhat difficult to interpret these new results because, at values of $[Ca^{2+}]_{added} > [EGTA]_{total}$ (or $[Ca^{2+}] \geq 1\ \mu M$), the true $[Ca^{2+}]_{free}$ values must be different from those reported, owing to the binding of Ca^{2+} to the

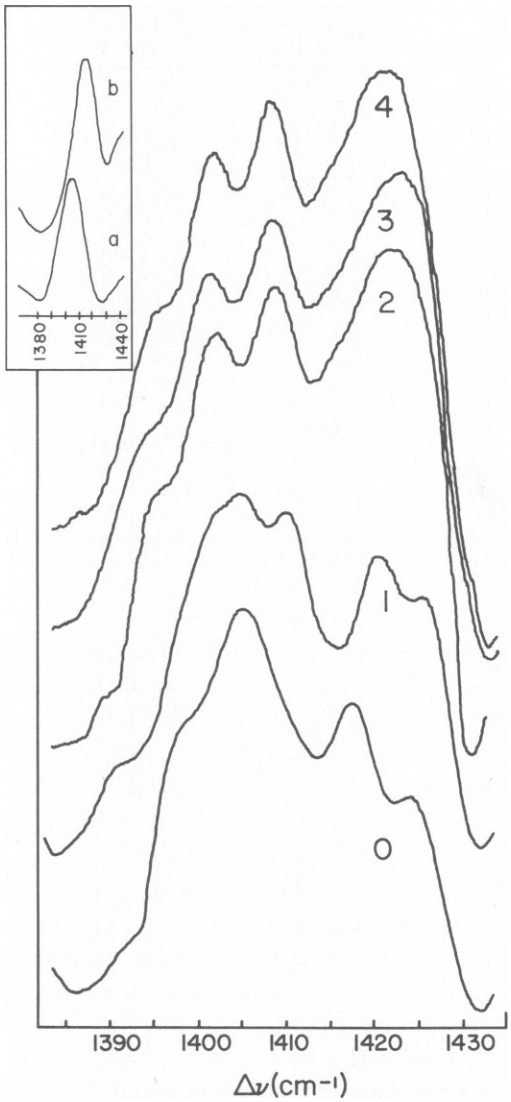

FIGURE 2 Expanded Raman spectrum showing region of CO_2^- symmetric stretch (1,403–1,411 cm^{-1}) and CH_2 side-chain deformation modes (1,418–1427 cm^{-1}). Conditions same as in Fig. 1. Inset: CO_2^- symmetric stretch of 10 mM EDTA without Ca^{2+} (*a*) and with 10 mM Ca^{2+} (*b*). EDTA in solution containing 0.1M KCl, 25 mM Hepes buffer, pH 7.4.

protein. Furthermore, stoichiometric calcium titrations at higher concentrations of TnC do not bring about large changes in CD and PMR spectra indicative of changes in secondary structure at $[Ca^{2+}]/[TnC] > 2$ (3, 7–9, 15, 25). Given these considerations, the absence of change in the laser Raman spectrum supports the view that calcium binding to the low affinity sites does not produce large secondary structural changes.

In addition to secondary structural features, several side-chain peaks that change upon calcium addition can be distinguished in TnC. Raman frequencies between 1,400 and 1,430 cm^{-1} are attributable to carboxylate symmetric stretch (1,405 cm^{-1}) and to CH_2 vibrations (1,423 cm^{-1}) of glutamic and aspartic acids and lysine residues. Several changes occur at these frequencies on addition of calcium to the first two sites (Fig. 2). First, the predominant peak in *apo* TnC (1,405 cm^{-1}) is split into peaks at 1,403 and 1,411 cm^{-1}. Comparison of this result with carboxylate frequencies of EDTA ± Ca^{2+} (Fig. 2 inset) suggests that the 1,403-cm^{-1} peak corresponds to ionized carboxylate groups, while that at 1,411 cm^{-1} is characteristic of carboxylates coordinated with calcium. The second set of peaks at 1,418 and 1,425 cm^{-1} merges to a single broad peak at 1,423 cm^{-1}.

It is somewhat puzzling that the carboxylate peaks do not show significant changes at $[Ca^{2+}]/[TnC] > 2$, since carboxylates are involved as Ca^{2+}-binding ligands in the sites of lower affinity, as well as in those of higher affinity. The lack of change beyond 2 Ca^{2+} may reflect the fact that carboxylate-containing residues in the lower affinity sites are in a highly structured environment even in the absence of bound metal, in contrast to those in the high affinity sites (7–9), thus imposing restrictions on symmetric stretch modes.

Tyrosine ring modes give rise to Raman peaks at ~ 853, 828, and 646 cm^{-1} (Figs. 3 and 4). The relative intensities of the first two have been shown (26) to be related primarily to the state of the phenolic OH; namely $I_{850}/I_{830} < 0.9$ in cases where a phenolic hydroxyl is

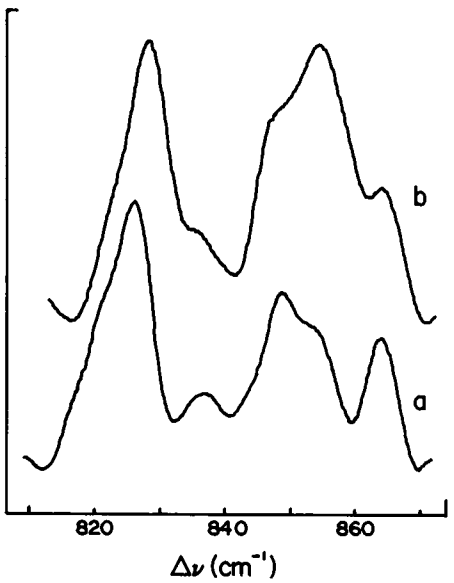

FIGURE 3 Expanded Raman spectrum of tyrosine ring modes. Conditions same as in Fig. 1. *Apo*-TnC (*a*), Ca_2^{2+} –TnC (*b*).

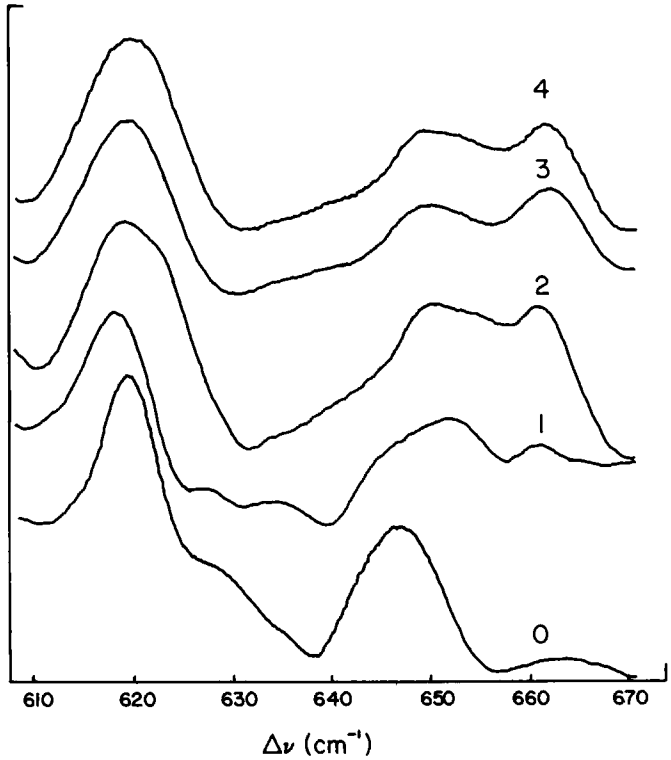

FIGURE 4 Expanded Raman spectrum showing phenylalanine ring twist (620 cm^{-1}), tyrosine ring twist (646–652 cm^{-1}), and C-S stretch (662 cm^{-1}). Conditions same as in Fig. 1.

H-bonded to a negatively charged acceptor, whereas H-bonding between an acidic donor and a phenolic oxygen generally yields $I_{850}/I_{830} > 0.9$. The ratio of 0.5 for the integrated intensities of these two peaks in *apo*-TnC (Fig. 3 *a*) indicates that the former case prevails, which is consistent with the fact that one of the two tyrosines, Tyr-109, is located within a Ca^{2+}-binding site rich in aspartic and glutamic acid residues available for H-bonding (27).

Binding two Ca^{2+} ions per molecule of TnC results in a shift of the I_{850}/I_{830} ratio to 1.0 (Fig. 3 *b*), suggesting that the acceptor of the phenolic proton is not available for hydrogen bonding in the Ca^{2+}-bound state. The 850/830 cm^{-1} doublet intensity ratio is also influenced by the degree of exposure of tyrosine residues to the solvent (26). In the case of TnC, however, this can be ruled out as contributing to the observed ratios, since tyrosine fluorescence quenching studies (unpublished; P. C. Leavis and S. S. Lehrer) indicate that the solvent exposure of both tyrosine residues (Tyr-10 and Tyr-109) is unaffected by Ca^{2+} binding.

The single symmetrical peak at 645 cm^{-1} corresponding to a tyrosine ring mode (Fig. 4) in the *apo*-TnC spectrum splits into two peaks at 646 and 652 cm^{-1} upon addition of the first two Ca^{2+} ions. This split is consistent with the view that of the two tyrosine residues in TnC, only Tyr-109 experiences a change in environment upon Ca^{2+} binding (6, 28).

The only other Ca^{2+}-induced change in the Raman spectrum occurs in the region of C-S stretch. The peak at 662 cm^{-1} is assignable to the single Cys residue in TnC (Cys-98). It intensifies severalfold upon the addition of the first two Ca^{2+} ions and then appears to broaden

slightly as the last two Ca^{2+} are added. Again the present results indicating a Ca^{2+}-induced change in the environment of Cys-98 are consistent with previous studies in which this cysteine residue either has been labeled with extrinsic probes or its reactivity as a function of Ca^{2+} concentration has been studied (5). The slight further change in the C-S stretch upon addition of the third and fourth Ca^{2+} ions indicates that low-affinity-site Ca^{2+} binding produces changes in the polypeptide chain in the vicinity of Cys-98. This is also suggested by a fluorescent probe on Cys-98 (5).

CONCLUSION

It is apparent that interpretation of Raman spectral changes in TnC is generally consistent with information derived using other techniques. The access to information about specific groups of residues in proteins afforded by Raman spectroscopy bodes well for its successful use in further studies on TnC and the other troponin subunits now under way in this laboratory.

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