# Spectroscopic Analysis of a Methionine-48 to Tyrosine Mutant of Chicken Troponin C†

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ABSTRACT: A mutant (M48Y) of chicken skeletal muscle troponin C was prepared in which Tyr replaced Met-48 of the recombinant protein (rTnC). Since Tyr and Trp are normally absent, spectral properties could be unambiguously assigned to the site of substitution. In the crystal structure, this residue lies at the COOH-terminal end of the B-helix of the N domain in a region postulated to undergo a significant conformational change to a more polar environment upon $Ca^{2+}$ binding [Herzberg et al. (1986) *J. Biol. Chem. 261*, 2638–2644]. Comparison of the far-UV CD spectra of M48Y and rTnC in the absence and presence of $Ca^{2+}$ indicated no overall structural alteration due to the mutation. However, $Ca^{2+}$ titration of the ellipticity change showed a reduction in $Ca^{2+}$ affinity and cooperativity of sites I and II. A $Ca^{2+}$-induced increase in the near-UV ellipticity of M48Y at pH 7.12 and a red shift in its UV absorbance spectrum occurred over a range of free [$Ca^{2+}$] attributable to the N-domain transition only. This was largely abolished at pH 5.3 where $Ca^{2+}$ no longer binds to sites I and II. That region of the $^1H$ NMR spectrum attributable to Tyr was broadened upon $Ca^{2+}$ binding. These $Ca^{2+}$-induced changes are consistent with the environment of the Tyr side chain becoming chiral, less polar, and more immobile, all in a direction opposite to that predicted. These observations indicate that while the general features of the postulated model are valid, it is unlikely to be correct in detail.

Troponin C (TnC)[1] is a calcium- and magnesium-binding protein of skeletal and cardiac muscles and a key element regulating contraction and relaxation of these tissues [for reviews, see Leavis and Gergely (1984), Grant (1985), and Zot and Potter (1987)]. The protein possesses four metal-binding sites, two of which are relatively high affinity and bind both calcium and magnesium. The remaining two are of lower affinity, bind only calcium ions, and are believed to be responsible for the regulation of contraction and relaxation. In vivo, TnC exists as a complex with the proteins troponins I and T. $Ca^{2+}$ ions released from the sacroplasmic reticulum interact with TnC to induce a conformational change which, in turn, communicates the "signal" for contraction to the other components of the regulated actomyosin system. Refined three-dimensional structures have been published for both turkey TnC (Herzberg & James, 1985, 1987; Herzberg et al., 1986 and the chicken protein (Sundaralingam et al., 1985; Satyshur et al., 1988). Both proteins show two distinct domains (N and C), one containing the two $NH_2$-terminal low-affinity $Ca^{2+}$-specific sites I and II, and the other the COOH-terminal high-affinity $Ca^{2+}$–$Mg^{2+}$ sites III and IV. The two domains are connected by an extended $\alpha$-helix so that the molecule has a dumbbell shape with minimal interdomain contact in the crystal structure. Since only high-affinity-binding sites are seen to be occupied by $Ca^{2+}$ ions in the crystals (grown at ~pH 5.0), the deduced structure is believed to represent TnC as it exists in resting muscle. A model has been presented to describe the calcium-induced conformational change of low-affinity sites I and II based on structural similarities between the unoccupied N-domain sites and occupied C-domain sites (Herzberg et al., 1986). This transition would involve a reorientation of helices flanking the binding loops and a partial exposure of some nonpolar side chains.

Recently, the application of protein engineering techniques to test the predictions of the model (Grabarek et al., 1990; Fujimoro et al., 1990; Pearlstone et al., 1992) has provided experimental support for its general features. In the present report, we have mutated a single Met-48 residue to Tyr. Since the chicken muscle and recombinant proteins are devoid of Tyr and Trp, this mutation permits the unambiguous assignment of spectral features to the environment of this aromatic residue. Residue 48 lies at the COOH-terminal end of the B-helix of the N domain and is one of those residues in the region postulated to undergo a $Ca^{2+}$-induced transition to a more polar environment at the surface of the molecule. However, several spectral properties indicate that the Tyr-48 side chain becomes less mobile and that its environment is less polar and becomes chiral in the $Ca^{2+}$-bound form. These observations indicate that while the general features of the model are almost certainly correct, it is unlikely to be valid in all details.

## EXPERIMENTAL PROCEDURES

*Construction of TnC Mutants and Protein Isolation.* The preparation of TnC mutants by site-directed mutagenesis and their expression in pLcIIFX have been described (Reinach & Karlsson, 1988; Fujimori et al., 1990; Golosinska et al., 1991; Pearlstone et al., 1992). The Met-48 to Tyr mutation was directed by a 19-base oligonucleotide containing sequence heterogeneity at the position corresponding to codon 48; 5′-TGATGAGG(T,G,A)(G,A)TCTGGGCCA-3′. M13 mutants were sequenced in their entirety to verify that adventitious mutations had not occurred. Replicative-form M13 bearing the M48Y mutation was digested with the restriction endonucleases *Bst*XI and *Sal*I for which there are unique sites. The fragment resulting from this digest contained

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[1] Abbreviations: TnC, troponin C; rTnC, recombinant troponin C; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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the coding region of TnC from codon 41 to the 3′ end. This was ligated into the expression vector pLcIIFX·TnC which had been similarly digested. Following transformation into QY13, clones were screened for inducible expression of fusion protein. Successful recombination of fragments of M13 DNA and expression vector was established by plasmid sequencing. The latter and also expression of the fusion protein and its purification and cleavage with factor Xa were as described previously (Golosinska et al., 1991).

*Spectral and Proton NMR Spectroscopic Analyses.* Preparation of buffers and stock protein solutions was as described previously (Golosinska et al., 1991; Pearlstone et al., 1992) with appropriate precautions for prevention of oxidation and contamination with $Ca^{2+}$. Final buffer compositions at pH 7.1 were 50 mM MOPS, 100 mM KCl, 1 mM EGTA, and 1 mM DTT; at pH 5.0, they were 50 mM sodium acetate, 100 mM KCl, 1 mM EGTA, and 1 mM DTT. Far- and near-UV CD analyses, difference absorbance measurements, protein concentration determinations, and calcium titrations using the previously reported binding constants were as described before (Golosinska et al., 1991; Pearlstone et al., 1992).

For $^1H$ NMR, 16 mg of M48Y was dissolved in 0.6 mL of 5 mM EDTA and 25 mM $NH_4HCO_3$ and applied to a gel filtration column (70 × 1.5 cm) of Sephadex G25 equilibrated with 25 mM $NH_4HCO_3$. Fractions from the leading shoulder were pooled, lyophilized, and dissolved in 670 μL of 100 mM KCl, 5 mM DTT, and 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer, pH 6.84, in $D_2O$. Protein concentration was 0.75 mM as determined by amino acid analysis. A Varian VXR 500 spectrometer was used with the following parameter settings: spectral width, 6000 Hz; pulse width, 86°; acquisition time, 1.6 s; relaxation delay, 1.4 s; 1024 scans; 30 °C. Titration was with 2-μL aliquots of 100 mM $CaCl_2$.

## RESULTS

*Far-UV CD studies.* The spectra for rTnC[2] and M48Y in the absence and presence of $Ca^{2+}$ are shown in Figure 1. Measured mean residue molar ellipticity values at 221 nm for both proteins in the apo state are similar (see legend to Figure 1) as is the increase in negative ellipticity upon addition of $Ca^{2+}$ to a pCa of 4.0. The values are similar to those previously reported for rTnC and the mutant F29W (Golosinska et al., 1991; Pearlstone et al., 1992) and for rabbit skeletal muscle TnC (Johnson & Potter, 1978; McCubbin et al., 1982). The results indicate that the structures of the two proteins are very similar and that the substitution of Met-48 by Tyr has little effect on the overall structural transition induced by $Ca^{2+}$.

Titration of the $\theta_{221nm}$ changes of rTnC and M48Y as a function of pCa (–log [$Ca^{2+}$]) is shown in Figure 2. While the midpoint of the transition for the high-affinity domain is not significantly affected by the mutation, that for the N domain is shifted to lower pCa (i.e., higher [$Ca^{2+}$]). The steepness of the curve for the N-domain transition is also significantly reduced. The parameters describing the biphasic curves were computed as described (Golosinska et al., 1991) and given in Table I. The data indicate that the substitution of Met-48 by Tyr leads to a decrease in $Ca^{2+}$ affinity of N-domain sites I and II and to a significant decrease in cooperativity between these two sites.

[2] As previously documented (Golosinska et al., 1991), this recombinant TnC (after cleavage with factor Xa) differs from chicken muscle TnC at two positions. The blocking moiety (probably acetyl) at the $NH_2$-terminus of the naturally occurring muscle protein is replaced by Met; Thr-130 in muscle TnC is replaced by Ile.

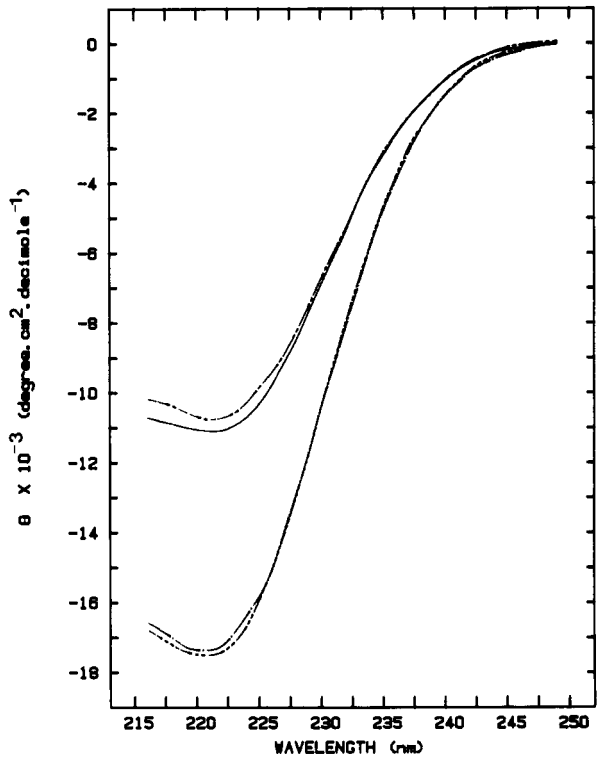


FIGURE 1: Far-UV CD spectra of rTnC and M48Y in the absence and presence of $Ca^{2+}$. Spectra were run in ±$Ca^{2+}$ as described under Experimental Procedures at pH 7.1. rTnC (–$Ca^{2+}$, —; +$Ca^{2+}$, – – –); M48Y (–$Ca^{2+}$, – · –; +$Ca^{2+}$, – · –). The data shown are the averaged spectra from four experiments for rTnC (~33 μM) and five experiments for M48Y (19–38 μM). The averaged initial and final values of $[\theta]_{221}$ (deg·cm²·dmol⁻¹) obtained in minus and plus $Ca^{2+}$ respectively, with standard deviations of the means in parentheses, were as follows: M48Y, –10 860 (±540), –17 230 (±690). Those for rTnC previously reported (Golosinska et al., 1991) were –11 160 (±960) and –17 560 (±800).

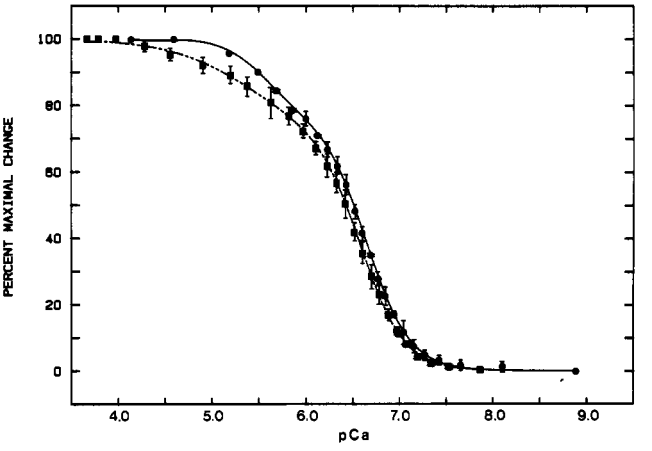


FIGURE 2: Far-UV CD calcium titration curves of rTnC (●) and mutant M48Y (■). The averaged data points from two titrations of rTnC and from four titrations of M48Y are shown by symbols. Standard deviation of the mean is indicated by a vertical bar. The calculated fitted curves (lines) for the averaged data sets for each protein were obtained as previously described (Golosinska et al., 1991). Protein concentrations were 32 μM for rTnC and 19–34 μM for M48Y in pH 7.1 buffer.

*Near-UV Circular Dichroism Studies.* The near-UV CD spectra of rTNC and M48Y in the absence and presence of $Ca^{2+}$ at pH 7.1 are shown in Figure 3A. The spectra of the two apoproteins show minima in ellipticity in the region of 250–270 nm associated with the major absorption bands of Phe. The spectra of both proteins in the absence of $Ca^{2+}$ in the region of 270–290 nm are featureless. In the case of rTnC,

Table I: Hill Coefficients and $-\log K$ Values for $Ca^{2+}$ Binding to rTnC and M48Y[a]

| | low-affinity sites | | high-affinity sites | |
|---|---|---|---|---|
| protein | $-\log K_2$ | $n$ | $-\log K_1$ | $n$ |
| rTnC | 5.65 ± 0.08 | 1.8 ± 0.2 | 6.67 ± 0.01 | 1.9 ± 0.1 |
| M48Y | 5.42 ± 0.14 | 1.1 ± 0.2 | 6.62 ± 0.04 | 2.0 ± 0.2 |

[a] The data sets for each of two titrations for rTnC and of four titrations for M48Y were analyzed separately using the curve-fitting program previously described (Golosinska et al., 1991). Deduced values of $-\log K$ and $n$ from these analyses were averaged and presented above with standard deviations.

this is as expected since neither Tyr nor Trp is present in the protein. The absence of ellipticity in the case of M48Y indicates that the Tyr side chain is in a nonchiral environment in the apoprotein state. Upon addition of $Ca^{2+}$, however, there is a sharp increase in the ellipticity of M48Y in the region of 270–290 nm, an increase not expected nor seen with rTnC. Figure 3B shows the difference CD spectra observed when $Ca^{2+}$ is added.

When these measurements with M48Y were repeated at pH 5.0, the apoprotein again showed almost no ellipticity in the 270–290-nm range (Figure 3A). Upon the addition of $Ca^{2+}$ to saturating levels, the increase observed at pH 7.1 is now virtually eliminated (Figure 3A,B). This was not unexpected since McCubbin et al. (1986) have shown that turkey TnC in solution binds only two $Ca^{2+}$ at pH 5.3 as compared with four at pH 6.8. Further, the X-ray structures of turkey and chicken TnC crystals prepared at pH 5.0–5.1 have shown that only the two high-affinity sites are occupied by calcium (Herzberg & James, 1988; Satyshur et al., 1988). Taken together, these observations indicate that the increased positive ellipticity seen at pH 7.1 upon $Ca^{2+}$ binding to M48Y can be ascribed to the structural transition of N domain only and that the environment of the Tyr side chain becomes chiral.

*Ultraviolet Difference Spectroscopy.* On the basis of far-UV CD titration data, three pCa values (7.77, 6.38, and 4.90) were chosen to examine calcium-induced UV absorbance spectral changes in the aromatic region. It was anticipated that at these concentrations of free calcium, all four calcium-binding sites would be unoccupied at pCa 7.77, the two high-affinity sites would be largely occupied at pCa 6.38, and all four sites would be occupied at pCa 4.90. The reference cell in each case contained proteins of identical concentrations in 1 mM EGTA.

The spectra (Figure 4) show that in going from a pCa of 7.77 where there is no spectral difference to a pCa of 6.38, there is a change attributable only to Phe with minima and maxima in the region of 250–275 nm. No perturbation of the Tyr absorbance in the region of 275–295 nm was detected. In contrast, a characteristic Tyr difference spectrum with positive peaks at 280 and 292.5 nm was observed when $Ca^{2+}$ was added to a pCa of 4.90. Further changes in the Phe spectral features were also observed.

These data clearly show that while the conformational transitions of both C- and N-terminal domains lead to changes in the environments of Phe residues (of which there are five in the C domain and six in the N domain), the spectral changes due to the single Tyr are associated only with the calcium-induced N-domain transition. The positive nature of this difference spectrum (Figure 4) indicates a red shift in the absorbance of the tyrosine side chain, normally interpreted as arising from a change to a more nonpolar environment (Donovan, 1969).

*Proton NMR Measurements.* To investigate the relative mobility of the Tyr side chain at position 48 as a function of

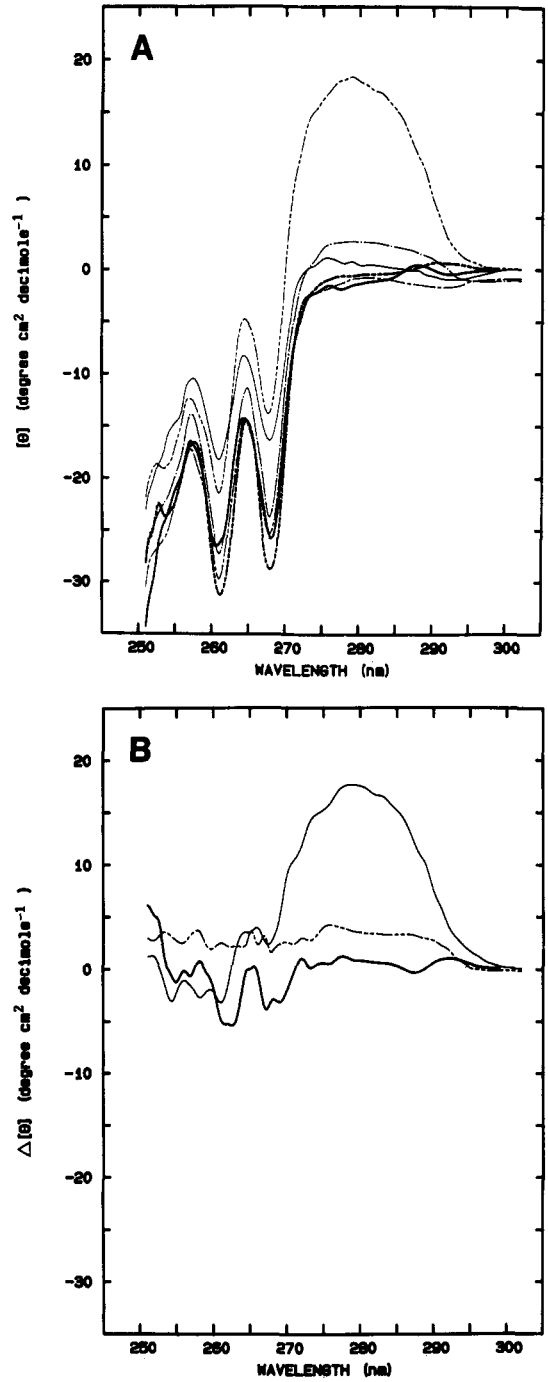


FIGURE 3: Effect of pH and $Ca^{2+}$ ions on the near-UV CD spectra of M48Y. (A) Near-UV CD spectra of rTnC and M48Y. At pH 7.1, rTnC ($-Ca^{2+}$, thick solid line; $+Ca^{2+}$, thick solid–dashed line); M48Y ($-Ca^{2+}$, —; $+Ca^{2+}$, – – –). At pH 5.0, M48Y ($-Ca^{2+}$, – – –; $+Ca^{2+}$, – · –). (B) Near-UV CD spectra ($\pm Ca^{2+}$) of rTnC and M48Y. At pH 7.1, rTnC (thick solid line); M48Y (thin solid line). At pH 5.0, M48Y (– – –). Protein concentrations were as follows: rTnC at pH 7.1, 103 μM; M48Y at pH 7.1, 182 μM; M48Y at pH 5.0, 125 μM.

occupancy of the binding sites by $Ca^{2+}$, $^1H$ NMR spectra were collected as described under Experimental Procedures. The portion of the aromatic region of the spectrum shown in Figure 5 shows the $C_4$ proton of His-128 as a singlet at 7.13 ppm, the $C_{3,5}$ protons (ortho to the OH group) of Tyr-48 as a doublet centered at 6.92 ppm, and other broader multiple resonances from protons of Phe residues. The doublet for the Tyr 3,5 protons is due to spin–spin coupling to the 2,6 protons. The Phe resonances are shifted upfield from their position in the absence of structure; they represent Phe side chains in the

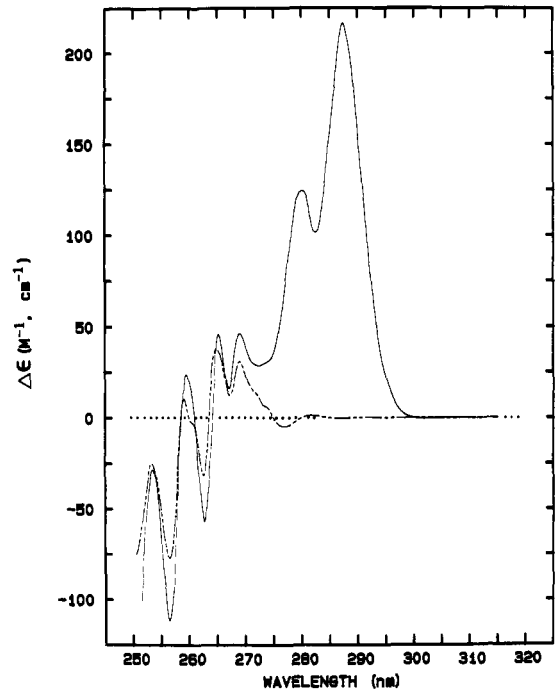


FIGURE 4: Difference absorbance spectra of M48Y at three concentrations of calcium in pH 7.1 buffer. Protein concentrations are as in Figure 1. Data were corrected for dilution and are expressed as the difference in molar extinction, $\Delta\epsilon$. Spectra were recorded after each of three successive additions of $CaCl_2$ to give final pCa values of 7.77 (···), 6.38 (– – –), and 4.90 (—).

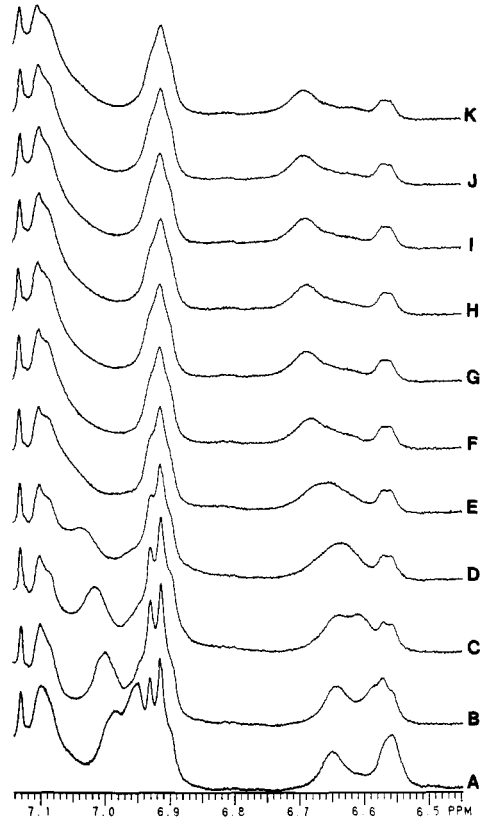


FIGURE 5: Portion (6.45 ppm → 7.15 ppm) of the aromatic region of the 500-MHz $^1H$ NMR spectra of M48Y presented as a function of added $Ca^{2+}$. These spectra are from a 0.75 mM sample of M48Y in 100 mM KCl, 20 mM Pipes, and 5 mM DTT, pH 6.84, in $D_2O$ at 30 °C. Each spectrum represents 1024 scans. Spectrum A represents the protein after treatment with EGTA and subsequent gel filtration chromatography as described in the text. The molar equivalents of added calcium chloride are 0.0, 0.38, 0.75, 1.13, 1.49, 1.88, 2.26, 2.63, 2.99, 4.13, and 5.28 for spectra A–K, respectively.

hydrophobic cores of the two domains of TnC. For example, the doublet at 6.56 ppm is from the δCH protons of Phe-151 (Tsuda et al., 1988). The spectrum shown in Figure 5A is typical for TnC with the $Ca^{2+}/Mg^{2+}$ sites occupied by $Ca^{2+}$ (McCubbin et al., 1982; Tsuda et al., 1988).

The doublet resonances corresponding to the 3,5 protons of the aromatic ring side chain of Tyr-48 are narrower than the resonances from the Phe residues. Since the line widths are proportional to the inverse of the rate of motion of the protons, this implies that the Tyr-48 side chain is more flexible in the state of the protein with only the high-affinity sites filled (spectrum A). While motion of the aromatic side chain around the Cβ–Cγ bond at rates greater than $\approx 10^3$ $s^{-1}$ is sufficient to make the 3 and 5 protons chemically equivalent as observed, this motion will not narrow the line width greatly since the axes of the 2–3 and 5–6 internuclear vectors responsible for relaxation are collinear with this axis. Thus, additional flexibility is required at a rate faster ($>10^8$ $s^{-1}$) than the rotational correlation time of the protein.

When $Ca^{2+}$ is added to the spectrum, several resonances move in the spectrum. These spectral changes take two calciums to complete and are indicative of calcium binding with off rate constants which are in the fast-exchange limit on the NMR time scale, as is typical for $Ca^{2+}$ binding to the low-affinity $Ca^{2+}$-specific sites of TnC. As $Ca^{2+}$ is bound, the doublet resonance corresponding to Tyr-48 3,5 protons broadens but does not change chemical shift. We interpret this to mean that Tyr-48 of M48Y is becoming more immobile as $Ca^{2+}$ is added to the $Ca^{2+}$-specific sites, which is consistent with the indication from other spectroscopic techniques that the aromatic ring of Tyr-48 is becoming more buried. The line broadening of the Tyr-48 3,5 protons as calcium is added is in contrast to the constant line widths observed for the His-128 C4 proton and the Phe-151 δCH protons. These resonances show neither line broadening nor chemical shift changes as calcium is added to the two low-affinity sites of TnC. Line broadening is observed for some resonances which show a chemical shift change as calcium is added. This line broadening is due to exchange broadening (Sykes & Scott, 1972) as indicated by the fact that it is the greatest for the resonances that shift the most, and indicates that the off rate for calcium is not that much greater than the chemical shift differences.

## DISCUSSION

The introduction of a single Tyr residue at position 48 of chicken skeletal TnC has permitted its use as a spectral probe for monitoring the change in environment of this residue during the $Ca^{2+}$-induced conformational transitions of the protein. Since there are no naturally occurring Tyr or Trp residues in the rTnC, the observed spectral properties could be unambiguously assigned to the mutated site.

An examination of the far-UV CD spectra of the two proteins, rTnC and M48Y, in the presence and absence of $Ca^{2+}$ indicated clearly that the global properties of the molecule were not significantly affected by the Y for M replacement. Thus, the mean residue molar ellipticity values for the two proteins are the same within experimental error and similar to those previously reported for the F29W mutant and for the rabbit skeletal muscle protein. It is also clear that the $Ca^{2+}$-induced Tyr spectral perturbations are specific for the N-domain transition. Thus, the near-UV CD changes involving an increase in positive ellipticity and attributable to Tyr were largely abolished when the pH was adjusted to pH

5.0 from 7.10. Under these more acidic conditions, binding of $Ca^{2+}$ to the low-affinity sites I and II does not occur (McCubbin et al., 1986). Further, while difference absorbance measurements at pCa values of 7.77, 6.38, and 4.90 showed changes in that portion of the UV spectrum attributable to Phe over this entire free [$Ca^{2+}$] range, those attributable to Tyr were restricted to pCa values between 6.38 and 4.90. Taken together, these observations provide convincing evidence that the spectral changes attributable to Tyr-48 are associated only with conformational transitions in the N domain and indicate little or no effect of the C-domain $Ca^{2+}$-induced structural changes on the environment of Tyr-48 in the mutated protein.

While the expectation that the insertion of Tyr at position 48 would prove to be a useful spectral probe of N-domain structure has proven to be correct, the nature and direction of the $Ca^{2+}$-induced spectral changes have been surprising. Residue 48 in the crystal structure of the two-$Ca^{2+}$ state is close to the COOH-terminal end of the B-helix of the N domain in a region whose side chains are postulated (Herzberg et al., 1986) to undergo significant conformational changes to a more polar environment upon transition to the four-$Ca^{2+}$ state. In the case of the naturally occurring Met-48, the side chain in the two-$Ca^{2+}$ state of the protein as deduced from the X-ray structure is largely exposed at the surface of the molecule with a solvent accessibility of 96 $Å^2$. Inspection of the crystal structure (two-$Ca^{2+}$ state) shows that the Tyr side chain can be sterically accommodated without local disruption of the immediate environment when Met is replaced. The spectral properties of the two-$Ca^{2+}$ state of M48Y (including lack of chirality and relatively high mobility of the aromatic side chain) are consistent with this relatively exposed position at the surface of the molecule. In the hypothetical model of the four-$Ca^{2+}$ state, this solvent accessibility is predicted to increase to 138 $Å^2$. The Met-48 and its Tyr replacement would have minimal interaction with neighboring residues, to be essentially fully accessible to solvent and mobile about its $C\beta$–$C\gamma$ bond. In light of the unrestricted environment of the side chain of Met-48 in the two-$Ca^{2+}$ state of the crystal structure and of the predicted four-$Ca^{2+}$ state, it seems unlikely that the present observations can be accounted for by a structural distortion induced by the replacement of Met-48 by Tyr. Such a distortion would require that the Tyr side chain assume a position very different from the fully exposed and mobile environment predicted for the naturally occurring side chain of Met-48 in the four-$Ca^{2+}$ state. Although this possibility cannot be categorically ruled out, it seems much more likely that the side chain of residue 48, whether it be Met or Tyr, becomes more buried in the transition to the four-$Ca^{2+}$ state. This is indicated by the development of aromatic positive ellipticity, a red shift in the UV absorbance spectrum as detected by difference spectrometry, and a broadening of that portion of the $^1H$ NMR spectrum attributable to Tyr. These changes are all in a direction opposite to that predicted by the model.

A possible alternative interpretation of the present observations, namely, that the side chain of Tyr-48 becomes more buried and immobile by virtue of the weak $Ca^{2+}$-induced dimerization of TnC, deserves consideration. Such dimerization has been documented by Margossian and Stafford (1982) and an apparent association constant of 511 $M^{-1}$ reported by Fujisawa et al. (1990) under buffer conditions similar to those used in the present work. On the basis of this value, it can be calculated that the percentage of dimer present at the concentrations employed in the present spectral measurements (33–182 $\mu$M) would be very low. Even at a concentration of 0.75 mM used in the $^1H$ NMR experiments, the percentage dimer would be less than 25%. Since line broadening in the NMR spectrum (see Figure 5 and Results) attributable to dimerization/aggregation was absent, we conclude that the burying and immobilization of the Tyr-48 side chain cannot be ascribed to such an effect.

A further feature of the present results with M48Y is the reduced $Ca^{2+}$ affinity of the low-affinity sites and a reduced cooperativity between these sites when compared with rTnC (see Table I). In light of the present observations that the side chain of residue 48 likely becomes more buried in going from the two-$Ca^{2+}$ to the four-$Ca^{2+}$ state, these effects are not surprising. The replacement of Met by Tyr would be expected to have a destabilizing effect on the four-$Ca^{2+}$ state but have little effect on the two-$Ca^{2+}$ state where the side chain is largely exposed at the surface. The net effect would therefore be to shift the equilibrium to the two-$Ca^{2+}$ state, resulting in a decreased affinity for $Ca^{2+}$.

The reduction in cooperativity between sites I and II in M48Y as compared with rTnC (and with the previously described F29W) is consistent with the effects of certain "hydrophobic patch" mutants whose $Ca^{2+}$-binding properties we have recently described (Pearlstone et al., 1992). Significant loss of site I and II cooperativity was also observed in several of these. In particular, M48A, involving the same Met residue mutated in the present work, showed a significant reduction in such cooperativity. In the present context, this becomes explicable in terms of a distortion of the structure of the four-$Ca^{2+}$ state by the replacement of a partially or fully buried nonpolar residue with one of much smaller side chain volume. These observations emphasize once again that even minor structural modifications of the N domain can lead to changes in site I and II cooperativity in the isolated protein.

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