# Helix Variants of Troponin C with Tailored Calcium Affinities†

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ABSTRACT: Muscle fiber contraction is regulated through calcium-induced changes in the conformation of troponin C. In this study, we explored the relationship between the stability of a specific helix in the protein and the metal ion affinity of associated binding sites. Serial replacement of the amino acid at position 130 caused the calcium affinity of the paired $Ca^{2+}/Mg^{2+}$ sites to be attenuated. In the crystal structures of chicken and turkey troponin C, position 130 is the N-cap residue of the G-helix. The ion affinities of variant proteins were shifted in the order Ile < Gly < Asp < Asn < Thr < Ser. Although differing in ion affinities, the variant proteins all exhibited high cooperativity. The results of this study point to a specific relationship between $\alpha$-helix stability and ion affinity in troponin C and suggest that troponin C may be a paradigm for protein folding problems.

Troponin C, a calcium- and magnesium-binding protein found on the thin filament of muscle fibers, regulates the contraction of skeletal and cardiac muscle by way of calcium-induced changes in conformation (Zot & Potter, 1987). Refined three-dimensional X-ray crystal structures exist for both turkey (Herzberg & James, 1988) and chicken (Satyshur et al., 1988) troponin C, and a model for the conformational change in troponin C that accompanies ion binding has been proposed (Herzberg et al., 1986). Genes encoding chicken (Reinach & Karlsson, 1988) and rabbit (Xu & Hitchcock-DeGregori, 1988) troponin C have been cloned, sequenced, and expressed in *Escherichia coli*, allowing for the creation of mutants (Trigo-Gonzalez et al., 1992) which facilitate the study of structure and activity relationships in this protein.

Crystal diffraction studies and circular dichroism (CD)[1] studies indicate that troponin C is a predominantly helical protein (Hincke et al., 1978; Herzberg & James, 1988; Satyshur et al., 1988). Moreover, CD studies indicate that the helical content of the protein is sensitive to calcium ions. We have chosen to examine, by site-directed mutagenesis, the relationship between the helicity of troponin C and its metal ion affinity. To do so, the stability of a single $\alpha$-helix in the carboxy-terminal domain of the protein was perturbed by substitutions at the helix N-cap (Richardson & Richardson, 1988). The consequences of substitution were observed by changes to the ion affinity of high-affinity sites.

## MATERIALS AND METHODS

Restriction endonucleases were purchased from New England Biolabs and Bethesda Research Limited. T7 DNA polymerase (Sequenase) was purchased from U. S. Biochemicals. All reagents were of the highest grade commercially available.

*Mutagenesis.* The DNA templates used in mutagenesis experiments all contained the substitution phenylalanine-105 to tryptophan (F105W) (Trigo-Gonzalez et al., 1992). Mutagenic oligonucleotides were prepared on an Applied Biosystems model 392 DNA/RNA synthesizer. Four oligonucleotides were used in the preparation of variant troponins C, one of which directed the substitution of an isoleucine codon for a threonine codon at position 130 in the mutageneic template (5′-G·CA·GTC·ACC·GAG·GAG·GAC-3′). The remaining three oligonucleotides contained sequence heterogeneities and were designed to direct the replacement of codon 130 with various amino acids:

(i) 5′-G·CAC·GTC·[A,G,C][A,G][C]·GAG·GAG·GAC-3′

(ii) 5′-G·CAC·GTC·[G,T][G,C,T][C]·GAG·GAG·GAC-3′

(iii) 5′-G·CAC·GTC·[G,C][A,C][T,A]·GAG·GAG·GAC-3′

Mismatches between the oligonucleotide and the M13 template are underlined. Mutagenesis was performed by the oligonucleotide-directed method (Zoller & Smith, 1982) as described for double-priming (Carter et al., 1985), with selection against template strands (Kunkel, 1985; Kunkel et al., 1987). Mutant genes were sequenced in their entirety by the chain termination method (Sanger et al., 1977) using the enzyme Sequenase (version 2.0). Mutant genes were subcloned into the expression vector pLcII-Fx-TnC as described previously (Trigo-Gonzalez et al., 1992).

*Expression and Purification of Troponin C Variants.* Troponin C was expressed and purified as described previously (Trigo-Gonzalez et al., 1992) with two modifications: (1)

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[1] Abbreviations: $A_{280}$,absorbance at 280 nm; CD, circular dichroism; C-terminal, carboxy terminal; DTT, DL-dithiothreitol; EGTA, ethylene glycol bis($\beta$-bisaminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; N-terminal, amino terminal; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TnC, troponin C; UV, ultraviolet.

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Factor $X_a$ cleavage (unnecessary in the context of this study) of the fusion proteins was omitted from the procedure so as to minimize the number of experimental manipulations and the likelihood of nonspecific proteolysis. (2) In place of the previously described HPLC step, proteins were purified to homogeneity by anion-exchange FPLC, following column chromatography in denaturing buffer (Trigo-Gonzalez et al., 1992). FPLC was performed on a Pharmacia FPLC system equipped with a Pharmacia HR10/10 Mono-Q column equilibrated in buffer A (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 1 mM $CaCl_2$, and 0.1 mM PMSF). Variant proteins were isolated in a linear gradient starting with buffer A plus 0.2 M NaCl and ending at buffer A plus 1.0 M NaCl. The variant proteins were precipitated from column fractions by the addition of ammonium sulfate to 50% (w/v). Precipitates were recovered by centrifugation and subsequently redissolved (generally 1–5 mg of protein) in 2.0 mL of buffer B (50 mM MOPS, pH 6.95, 150 mM KCl, and 1.0 mM EGTA) containing 3.0 mM DTT and 8 M urea. Aliquots of each variant were transferred to separate dialysis bags, and the six protein samples used in this study were then dialyzed simultaneously, in the same plastic vessel, for 30 h at 4 °C, against several changes of buffer B plus 1.0 mM DTT. Protein concentrations were determined by a modification (Markwell et al., 1981) of the method of Lowry et al. (1951) and samples were analyzed for the presence of residual calcium by atomic absorbance spectroscopy.

*Spectroscopy.* Absorbance, fluorescence, CD spectra, and calcium titrations of the spectra of variant proteins were recorded essentially as described previously (Trigo-Gonzalez et al., 1992). Far-UV CD measurements were conducted at 25 °C on a Jasco J-710 spectropolarimeter fitted with a thermostated cell holder. Protein samples were diluted in buffer B containing 1.0 mM DTT to concentrations between 7.86 and 10.63 $\mu$M. CD spectra were recorded in a cell with a path length of 0.1 mm both before and after addition of calcium to a final free calcium concentration of 0.5 mM. Fluorescence spectra and titrations were recorded on a Photon Technology Model LS-100 fluorometer equipped with a thermostated cell assembly and interfaced with a microcomputer. Samples were maintained at 25 °C. Fluorescence analyses were performed on protein samples which produced an $A_{280} < 0.05$ (approximately 5 $\mu$M protein), in 1.0 $cm^2$ quartz cuvettes. Fluorescence titrations were performed by the incremental addition of calcium chloride to samples prepared as described for spectra. Samples were excited at 278 nm, and the fluorescence emission was recorded at 366 nm.

## RESULTS

*Mutagenesis.* A total of nine different mutants were isolated using the three mutagenic oligonucleotides described under Materials and Methods. These included the substitutions at position 130 of Asn, Asp, Glu, Gly, His, Phe, Ser, Thr, and Val. For the purposes of this study, only six position 130 variants were expressed and characterized (Ile, Gly, Asp, Asn, Thr, Ser), including the wild type, Ile-130 (Golosinska et al., 1991). Each of the variants was created in such a way that a tryptophan reporter group was present at position 105 (Figure 1). Accordingly, clones were named F105W/I130, F105W/I130G, F105W/I130D, F105W/I130N, F105W/I130T, and F105W/I130S.

*Expression and Purification.* In previous studies, fusion proteins prepared from the pLcII-Fx-TnC expression vector were processed with the enzyme Factor $X_a$ to remove a 38-residue amino-terminal peptide (Nagai & Thogersen, 1987). Preliminary studies with the recombinant wild-type fusion protein indicated that Factor $X_a$ digestion was unnecessary for the function of the recombinant TnCs. Therefore, the step was omitted from purification protocols. All subsequent analyses were conducted with fusion proteins.

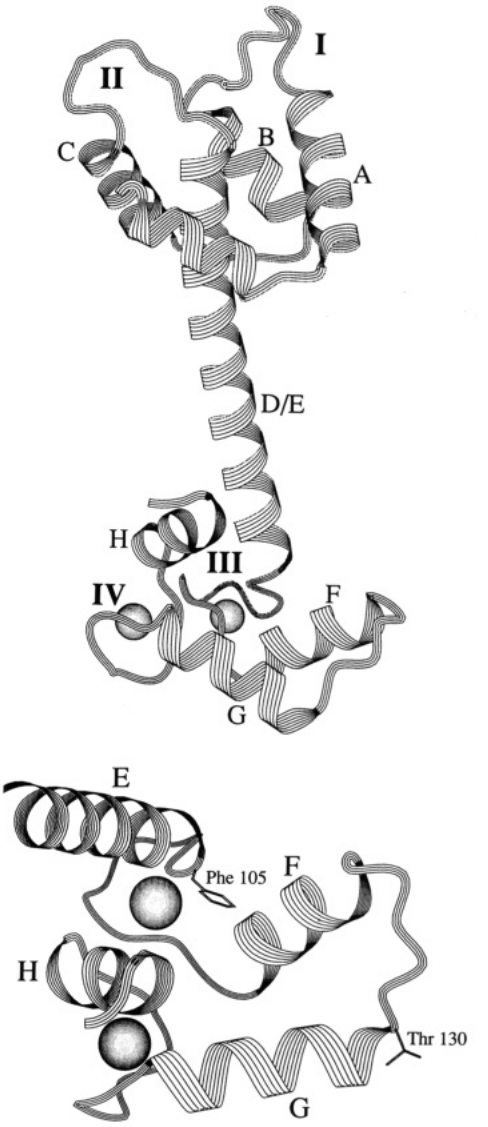


FIGURE 1: Ribbon structures of native chicken skeletal troponin C. (A, top) Main chain structure of the entire protein; (B, bottom) isolated view of the high-affinity (C-terminal) domain. Low-affinity, $Ca^{2+}$-specific sites I and II are found in the N-terminal domain, and high-affinity $Ca^{2+}/Mg^{2+}$ sites III and IV are found in the C-terminal domain. Helices are labeled A–H, the calcium ions bound to sites III and IV are represented as spheres, and the side chains of amino acid residues at positions 105 and 130 are indicated. Under the conditions of low pH necessary for crystallization of troponin C (Herzberg & James, 1985; Sundaralingam et al., 1985), each helix is seen fully formed, and low-affinity sites are unoccupied by metal ions. This ribbon image was generated from the $C_\alpha$ coordinates of chicken TnC (Satyshur et al., 1988) from Brookhaven file 4TNC (Bernstein et al., 1977), using the modified Ribbon programs (Priestle, 1988; Callahan et al., 1990).

The final step in purification of the fusion proteins involved anion-exchange FPLC in a 1 mM calcium buffer, replacing the previous HPLC method. The higher capacity of the anion-exchange column afforded greater yields of protein than HPLC with equivalent purity. All protein samples were judged to be homogeneous by SDS–PAGE and, after dialysis, were shown to be calcium-free, by atomic absorbance spectroscopy (Trigo-Gonzalez et al., 1992).

Table I: Summary of Physical Data for N-Cap Variants of Troponin C[a]

| mutant | all sites $\Delta[\theta]_{221}$ ($\times 10^{-3}$) (deg·cm$^2$·dmol$^{-1}$) | high-affinity sites −log $K_d$ | high-affinity sites $\Delta\Delta G_{Ile \rightarrow Mut}$ (kcal·mol$^{-1}$) | high-affinity sites Hill coefficient |
|---|---|---|---|---|
| F105W/I130 | −5.4 ± 0.1 | 7.462 ± 0.013 | 0.00 | 2.1 ● 0.1 |
| F105W/I130G | −5.8 ± 0.8 | 7.608 ● 0.009 | 0.20 ± 0.02 | 2.1 ± 0.1 |
| F105W/I130D | −5.3 ± 0.6 | 7.691 ± 0.011 | 0.31 ± 0.02 | 1.9 ± 0.1 |
| F105W/I130N | −5.3 ± 0.3 | 7.752 ● 0.011 | 0.40 ± 0.02 | 1.8 ± 0.1 |
| F105W/I130T | −4.4 ± 0.9 | 7.943 ● 0.012 | 0.66 ± 0.02 | 1.7 ± 0.1 |
| F105W/I130S | −5.1 ± 0.9 | 7.945 ± 0.012 | 0.66 ± 0.02 | 2.0 ± 0.1 |

[a] Far-UV CD measurements were performed on a Jasco J-710 spectropolarimeter as described under Materials and Methods. Protein samples varied in concentration from 7.86 to 10.63 μM. CD spectra were recorded in a cell with a path length of 0.1 mm before and after adding $CaCl_2$ to a final free-calcium concentration of 0.5 mM. The values represent the mean change (±SD) in $[\theta]_{221}$ for 2–5 determinations and represent contributions from both high- and low-affinity domains of the proteins. Fluorescence titration data, as shown in Figure 3, were fitted to an equation for a single class of binding sites as follows: $y = (K_a[Ca^{2+}])^h 100/[1 + (K_a[Ca^{2+}])^h]$, where the percentage saturation is given by $y$, $K_a$ is the equilibrium constant for association ($1/K_d$), and $h$ is the Hill coefficient. Each variant was titrated twice, and the data were fitted by an iterative, derivative-free, curve-fitting routine (BMDP Statistical Software, Inc.) on an IBM 3081 computer. The contribution of individual amino acids to the free energy of binding is given relative to the free energy change for the binding of calcium to the isoleucine-130 variant; $\Delta\Delta G_{Ile \rightarrow Mut} = (-RT \ln K_d^{Mut}) - (-RT \ln K_d^{Ile})$.

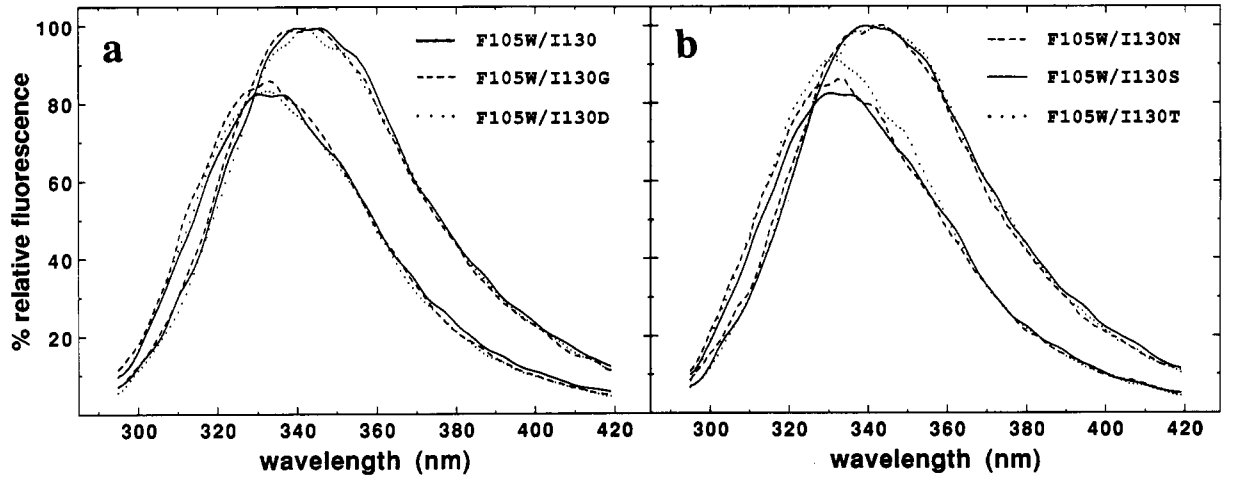


FIGURE 2: Overlaid fluorescence emission spectra of fusion troponins C: (a) F105W/I130, F105W/I130G, F105W/I130D; (b) F105W/I130N, F105W/I130S, F105W/I130T. Protein samples were diluted to a final concentration of 0.5 μM in buffer B (50 mM MOPS, pH 6.95, 150 mM KCl, and 1.0 mM EGTA) containing 1.0 mM DTT, and spectra were recorded before and after the addition of $CaCl_2$ to a final free calcium concentration of 0.5 mM. Spectra were obtained as described under Materials and Methods, at an excitation wavelength of 278 nm. Apoprotein samples (0.5 μM, $A_{280} \leq 0.05$) produced fluorescence maxima centered near 345 nm whereas the calcium-saturated proteins had maxima near 330 nm.

*Spectroscopy.* Far-UV CD spectra of variant proteins were recorded before and after the addition of calcium chloride to a final free calcium concentration of 0.5 mM. The spectra of the six apoproteins (not shown) were similar to one another and similar in their response to calcium, within the error of the method. The absolute changes in ellipticities for each of the proteins are shown in Table I and are the average of two to five separate experiments. No significant differences were detected by far-UV CD between the helical content of the six apoproteins, nor were significant differences detected in the helical content of the six calcium-saturated proteins.

The fluorescence emission spectra of the six proteins are shown in Figure 2. The spectra were characteristic of the tryptophan residue at position 105 (Trigo-Gonzalez et al., 1992), and the same calcium-induced changes in spectra were observed for each of the variant proteins. Magnesium-induced changes in the fluorescence spectra were also determined (data not shown). As was observed in the calcium-induced spectra, there were no significant differences when the six variants were compared. The tryptophan reporter group behaved as previously reported.

*Titration of Tryptophan Fluorescence.* Significant differences in the calcium titration of mutant proteins were observed. The results of calcium titrations are shown in Figure 3. Serine- and threonine-substituted proteins had the highest affinities for calcium followed by, in order of decreasing affinity, asparagine, aspartic acid, glycine, and isoleucine. When expressed in terms of a free energy change, the difference between the highest affinity variant and lowest affinity variant was 0.66 ± 0.02 kcal·mol$^{-1}$. The Hill coefficients extracted from a fit of the binding curves were high for all of the proteins, varying between 1.7 ± 0.1 and 2.1 ± 0.1. The Ile and Thr fusion proteins analyzed in this study had dissociation constants slightly lower than those values reported for naturally occurring Ile and Thr variants. However, in the two studies, the relative difference in the binding constants of Ile- and Thr-substituted proteins was the same; ΔpCa = 0.481 ± 0.13 (Table I) versus ΔpCa = 0.4 ± 0.1 (Golosinska et al., 1991; Trigo-Gonzalez et al., 1992).

## DISCUSSION

Troponin C (TnC) has four metal ion-binding sites (Figure 1A). Two of these sites, so-called $Ca^{2+}$-specific, occupy the amino-terminal domain of the protein and have a relatively low affinity for calcium. The remaining two sites occupy the carboxy-terminal domain, have a high affinity for calcium, and can alternatively bind magnesium (Zot & Potter, 1987). Hence, the sites in the C-domain are so-called $Ca^{2+}/Mg^{2+}$-sites (Figure 1B). When troponin C is not complexed with other proteins of the thin filament, the low- and high-affinity sites are seen to titrate independently of one another (Trigo-Gonzalez et al., 1992).

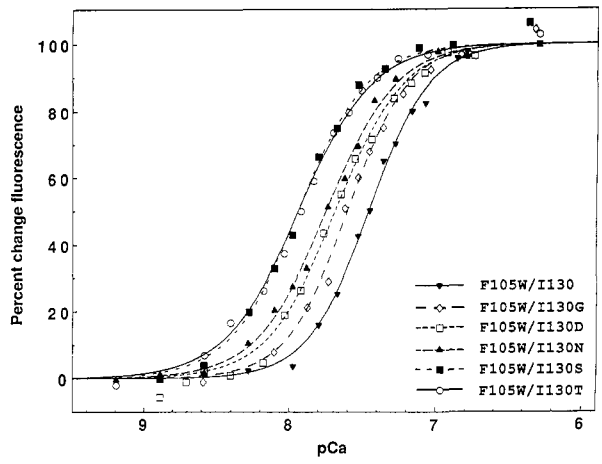


FIGURE 3: Titration of tryptophan-105 fluorescence in helix variants. Titrations were performed as before (Trigo-Gonzalez et al., 1992) with experimental conditions identical to those described in Figure 2. Titration data are plotted as a percentage of the absolute change in fluorescence intensity. Binding parameters are presented in Table I. The fluorescence excitation wavelength was 278 nm, and emission was recorded at 366 nm. Free calcium ion concentrations were calculated from total calcium in the chelating buffer by a computer algorithm (Perrin & Sayce, 1967) adapted to the MacIntosh microcomputer by Dr. B. Sykes and Mr. J. Boyko (Department of Biochemistry, University of Alberta).

Intensive efforts have been made to identify the structural features of troponin C which give the protein its characteristic ion affinities and ion specificities. For example, it is known from peptide studies that the number and arrangement of charged ligands surrounding a bound metal ion will contribute to the affinity of an individual binding site. Marsden et al. (1988) showed, using a series of peptide analogues to troponin C's site III, that maximum affinity was achieved when the negatively charged side chains of liganding residues were present at positions that were axial to one another as opposed to adjacent to one another. Similar results were reported by Reid (1990) albeit with larger analogues.

It is well established that some relationship exists between metal ion binding and the helicity of troponin C. Calcium-induced changes in the helical content of TnC have been observed in the far-UV CD spectrum of the protein (Hincke et al., 1978). The $Ca^{2+}/Mg^{2+}$ sites contribute approximately 65% to the overall change in ellipticity whereas $Ca^{2+}$-specific sites contribute approximately 35%. From CD studies of rabbit skeletal troponin C (159 amino acids), it has been estimated that the number of amino acid residues participating in helices increases from roughly 55 residues to 77 residues when the $Ca^{2+}/Mg^{2+}$ sites are saturated. The saturation of both $Ca^{2+}/Mg^{2+}$ sites and $Ca^{2+}$-specific sites is estimated to increase the helical content of the protein to 87 residues (Reid et al., 1981).

The relationship between helicity and ion affinity was underscored further by studies involving peptide analogues and fragments of troponin C (Reid et al., 1980, 1981). Peptide fragments were generated which encompassed the site III binding loop and varying lengths of sequence flanking the loop. It was found that the ion affinity of the peptides was increased according to the length of the helix on the amino terminal side of the loop. Surprisingly, the length of the helix on the carboxy-terminal side of the loop had a negligible influence on binding affinity [reviewed by Marsden et al. (1990)].

We predicted that amino acid substitutions at position 130, the N-cap of TnC's G-helix (Herzberg & James, 1988; Satyshur et al., 1988), would have a stabilizing or destabilizing influence on the helix and that this influence would be reflected in changes to the ion affinity of the $Ca^{2+}/Mg^{2+}$ sites (Figure 1B). Our prediction had support from mutagenesis studies which showed that the amino acid forming the first helical residue (Presta & Rose, 1988), or N-cap (Richardson & Richardson, 1988), of an $\alpha$-helix has a large influence on the stability of the associated helix. N-cap residues in the enzymes barnase (Serrano & Fersht, 1989) and T4 lysozyme (Nicholson et al., 1988) contribute to the position of equilibria between folded and unfolded states of these proteins and, by analogy, we anticipated that N-cap residues in troponin C would help determine the equilibrium between metal-bound and metal-free conformations. The results of a study which compared the ion affinities of natural variants of TnC (Golosinska et al., 1991) lent further support to our prediction. In this case, it was shown that the calcium affinity of $Ca^{2+}/Mg^{2+}$ sites was 4 times greater when threonine occupied position 130 than when isoleucine occupied position 130.

Six variants of TnC were created and characterized. Each contained a primary amino acid substitution at position 105 (Phe-105→Trp) (Trigo-Gonzalez et al., 1992) and a secondary amino acid substitution at position 130 (one of Ile, Gly, Asp, Asn, Thr, or Ser) (Figure 1B). Chicken troponin C does not naturally contain either Trp or Tyr residues (Reinach & Karlsson, 1988). Therefore, high-affinity metal-binding events were monitored unambiguously by changes in the intrinsic fluorescence of the Trp-105 proteins (Figure 2). Moreover, since the Trp-105 reporter group is sensitive to changes in the conformation of the high-affinity sites (for example, Trp-105 responds differentially to calcium and magnesium ions; Trigo-Gonzalez et al., 1992), we considered fluorescence spectra to be diagnostic of gross aberations in the structure of the C-terminal domain. The spectroscopic evidence suggests that the substitutions at position 130 had no effect on the overall structure of the C-domain. The spectra shown in Figure 2a and Figure 2b were superimposable as were the fluorescence spectra of magnesium-saturated proteins (data not shown). The apoproteins also had similar far-UV CD spectra and underwent similar calcium-induced changes in the far-UV CD spectra (Table I). Presumably, the structural consequences of mutation were restricted to the vicinity near the N-cap of helix G. The only measurable consequences of amino acid substitutions at position 130 were changes in the ion affinity of $Ca^{2+}/Mg^{2+}$ sites (Figure 3 and Table I). Residues that were capable of forming a hydrogen bond with the mainchain >N–H (Thr, Ser, Asn, and Asp) conferred higher affinities (lower dissociation constants) on the variant proteins than residues unable to form such bonds (Gly and Ile).

The process by which troponin C cooperatively binds calcium ions involves multiple conformational states of the protein and differing occupancies of the four ion-binding sites. Consequently, the system can be described by several models which vary in their degree of complexity. Nevertheless, to a first approximation, troponin C (or at least the high-affinity sites in troponin C), may be considered a simple three-state folding system (Scheme I) in which there is equilibrium between (1) an unfolded state (U), as may exist in the presence of denaturants; (2) a partially folded but calcium-free state ($F_{-Ca}$); and (3) a fully folded and calcium-loaded state ($F_{+Ca}$).

Scheme I

$$U \rightleftharpoons F_{-Ca} \rightleftharpoons F_{+Ca}$$

The results of the current study do not allow us to determine the degree to which a mutation perturbs the free energy of a particular folding state. Calcium titrations only provide us

with information regarding the difference in the free energies of the $F_{-Ca}$ and $F_{+Ca}$ states. Hence, shifts observed in the calcium affinity of mutant proteins indicate changes in the free energy of either the $F_{-Ca}$ state or the $F_{+Ca}$ state, or possibly some combination of changes in both states. Despite our inability to correlate the shifts in ion affinity with changes to a particular folding state, a reasonable interpretation of the results can be made if we assume that the mutations made in this study have their influence primarily on the fully folded or $F_{+Ca}$ state. This assumption is the basis of the discussion that follows. In future studies, we propose to quantitate the contribution of individual mutations to a particular folding state of the protein. To this end, we are examining the equilibrium between unfolded (U) states and folded states (F) by established methods that involve urea denaturation (Horovitz et al., 1992; Serrano et al., 1992).

In a survey of crystal structures, Richardson and Richardson (1988) found that Asn, Ser, and Asp are preferred N-cap residues. However, from the mutagenesis studies described here and elsewhere (Bell et al., 1992), it is clear that the most appropriate choice depends on the context of the N-cap residue within the overall structure. Notably, Asp and Asn were not as effective at stabilizing the bound conformation of TnC as Thr and Ser. The difference in the quality of Asp versus Asn may be explained by the high concentration of negative charge at the N-terminal end of the G-helix (the tripeptide sequence following position 130 is Glu-Glu-Asp), which would give rise to destabilizing charge-repulsion interactions in the Asp variant. Ile has a bulky, aliphatic side chain that cannot form a hydrogen bond back to the main chain of the protein. Therefore, it most likely destabilized the N-terminal end of the G helix and was a poor N-cap residue. Gly is also unable to form a hydrogen bond to the main chain. Yet, because Gly lacks a bulky side chain, it may provide greater stability to the helix then Ile simply by allowing a water molecule to hydrogen bond with the main chain donor.

Thr and Ser variants, in which the side chains differ only by the $\gamma$-methyl group of the threonine, give dissociation constants ($K_d$'s) that are nearly indistinguishable. This is not surprising since, from our examination of crystal structures, the $\gamma$-methyl of threonine does not participate in hydrophobic interactions with surrounding parts of the protein. By comparing sequences of natural TnCs (EMBL/SwissProt protein and nucleotide sequence sets), we have found that a Thr or Ser residue always initiates the first helix of a helix–loop–helix binding site. The sole exception to this rule is the Ile residue found at position 130 of recombinant chicken TnC (Reinach & Karlsson, 1988; Golosinska et al., 1991). Although the current study indicates a relationship between ion affinity and the stability of certain helices, it seems that nature has not extensively varied the N-cap residue in order to fine-tune ion affinities. Nevertheless, the connection between helix stability and ion affinity may be exploited by variation in sequences which are internal to the helices. The mechanism relating helical content and stability to ion-binding affinity is not known. However, we speculate that it involves interactions between the natural dipole (Shoemaker et al., 1987) of the G-helix and charged residues in the binding loop. Consistent with this interpretation, apo-TnC becomes as helical as the calcium-saturated TnC when the carboxylates in the binding loops are protonated at low pH (Hincke et al., 1978; McCubbin et al., 1982).

Perhaps the most remarkable observation of this study was the high value of Hill coefficients derived from binding curves. These approached the theoretical limit of 2 and did not vary greatly from variant to variant (Table I). Apparently, position 130 substitutions have little effect on the degree of cooperativity between the high-affinity sites. This observation adds support to the three-state model of folding/conformational change in troponin C (Scheme I) and the notion that paired sites are functionally linked (Teleman et al., 1983; Iida, 1988). It is difficult to explain why the contributions of individual amino acids to the free energy of binding, $\Delta\Delta G$ (Table I), were generally smaller than contributions reported for the free energy of folding in helix variants of barnase and T4 lysozyme. However, it is notable that the order of stabilities in the TnC variants (Ile < Gly < Asp < Asn < Thr < Ser) is similar to the order of stabilities in N-cap variants of T4 lysozyme at pH 6.5 [Gly < (Val, Ala) < Asp < Asn < Ser] (Bell et al., 1992).

In many respects, the equilibrium between metal-bound and metal-free conformations of troponin C is analogous to the equilibrium which exists between the folded and unfolded states of all proteins. A fully folded protein is more highly structured than its folding intermediates in the same way as the calcium-saturated troponin C is more highly structured than calcium-free troponin C. Accordingly, we viewed metal-free conformations of TnC as intermediates on a pathway (Matouschek et al., 1989) to a fully folded, calcium-saturated TnC. We anticipated that the structural features of TnC which determine the equilibrium between metal-bound and metal-free conformations and, hence, ion affinity would be the same as the structural features that determine equilibria between the folded and unfolded states of proteins generally.

In summary, this study has shown that there is a specific relationship between the stability of helix G in troponin C and the ion affinity of $Ca^{2+}/Mg^{2+}$ sites. Relationships of this nature may be a general property of proteins with helix–loop–helix structural motifs, used to fine-tune ion affinities.

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