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Articles

A Comparative Spectroscopic Study of Tryptophan Probes Engineered into High- and Low-Affinity Domains of Recombinant Chicken Troponin C†

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ABSTRACT: The spectral properties of three tryptophan-substituted mutants of recombinant chicken troponin C are compared. Site-specific mutagenesis was used to introduce a tryptophan residue into the high-affinity (Ca^{2+}/Mg^{2+}) domain of troponin C at residue position 105, thereby creating the mutant phenylalanine-105 to tryptophan (F105W). The spectral properties of F105W and a double mutant, F29W/F105W, were compared with the mutant phenylalanine-29 to tryptophan (F29W). Since wild-type chicken troponin C does not naturally contain either tyrosine or tryptophan residues, the tryptophan substitutions behaved as site-specific reporters of metal ion binding and conformational change. The residues that occupy positions 29 and 105 are at homologous locations in low-affinity and high-affinity domains, preceding the first liganding residues of binding loops I and III, respectively. Mutant proteins were examined by a combination of absorbance and fluorescence methods. Calcium induced significant changes in the near-UV absorbance spectra, fluorescence emission spectra, and far-UV circular dichroism of all three mutant proteins. Magnesium induced significant changes in the spectral properties of only F105W and F29W/F105W proteins. Tryptophan substitutions allowed Ca^{2+}-specific and Ca^{2+}/Mg^{2+} sites to be titrated independently of one another. Results indicate that there is no interaction between the two binding domains under conditions where troponin C is isolated from the troponin complex. Magnesium-induced changes in the environment of the tryptophan reporter at position 105 were significantly different from those induced by calcium. This suggests that calcium and magnesium differ in their influence on the conformation of the high-affinity, Ca^{2+}/Mg^{2+} sites.

Troponin C (TnC) is a calcium binding protein of skeletal and cardiac muscle and the key element regulating contraction of these tissues. Troponin C has four metal binding sites, two of a relatively high affinity ($K_a \simeq 10^7\ M^{-1}$) that are capable of binding either calcium or magnesium ions, referred to as the Ca^{2+}/Mg^{2+} sites, and two sites of low affinity ($K_a \simeq 10^5\ M^{-1}$) which bind only calcium ions, the Ca^{2+}-specific sites. Three-dimensional X-ray crystal structures of both turkey (Herzberg & James, 1985) and chicken troponin C (Satyshur et al., 1988) have been published. The structures revealed by crystal diffraction studies are organized into two domains separated by a long interdomain α-helix and therefore have a "dumbbell"-shaped appearance. Ca^{2+}-specific sites inhabit the amino-terminal domain of the protein, and Ca^{2+}/Mg^{2+} sites are found in the carboxyl-terminal domain.

Genes encoding chicken (Reinach & Karlsson, 1988) and rabbit (Xu & Hitchcock-DeGregori, 1988) troponin C have been cloned, sequenced, and expressed in *Escherichia coli*, and consequently, it has been possible to examine relationships between the fine-structure and activity of troponin C by site-specific mutagenesis of the cloned genes (Pearlstone et al., 1992; Grabarek et al., 1990; Fujimori et al., 1990; Dobrowolski et al., 1991). We report the comparative spectral

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analysis of a family of tryptophan-substituted mutants of troponin C. These mutants are designed to report metal ion binding events in both Ca^{2+}/Mg^{2+} sites and Ca^{2+}-specific sites and, hence, to facilitate structure/activity studies by mutagenesis. Three tryptophan-substituted mutants are characterized and compared, one of which, phenylalanine-29 to tryptophan (F29W), is described elsewhere (Pearlstone et al., 1992).

A variety of methods are available for studying the interactions of proteins with their ligands. Binding interactions that bring about changes in the environment of chromophores, in either the protein or the ligand, can be observed spectroscopically (Donovan, 1969). Often, however, suitable chromophores are not available in proteins, or, when available, they are not at positions that will be influenced by binding. In such circumstances, extrinsic reporters may be added to the system (Cantor & Schimmel, 1980).

Binding constants for Ca^{2+}/Mg^{2+} sites in TnC have been estimated by equilibrium dialysis (Potter & Gergely, 1975), by titration of tyrosine fluorescence (in rabbit skeletal troponin C) (Johnson & Potter, 1978), and by titration of the far-UV ellipticity of the protein (Hincke et al., 1978; Johnson & Potter, 1978). Binding constants for low-affinity Ca^{2+}-specific sites in TnC have been more difficult to estimate because suitable intrinsic reporters are unavailable and because calcium binding at low-affinity sites is only responsible for about 30–40% of the change in far-UV ellipticity associated with calcium binding. Extrinsic probes have been used to titrate low-affinity calcium binding to TnC (Johnson et al., 1978; Zot & Potter, 1987b; Grabarek et al., 1990).

Tryptophan substitutions by mutagenesis are an alternative to chemical methods for the introduction of reporter groups into TnC. Chicken troponin C does not naturally contain either tyrosine or tryptophan residues, and, therefore, the tryptophan residues in mutants of TnC behaved as site-specific reporters of calcium and magnesium ion binding and of conformational changes.

MATERIALS AND METHODS

Restriction endonucleases were purchased from New England Biolabs and Bethesda Research Limited. The Klenow fragment of DNA polymerase I was purchased from Boehringer Mannheim, and T7 DNA polymerase (Sequenase) was purchased from U.S. Biochemicals. All chemicals and reagents were of the highest grade commercially available.

Expression and Purification of Recombinant Troponin C and Mutants. Recombinant chicken troponin C was expressed in *Escherichia coli* from a gene carried on the vector pLcII-Fx-TnC (Nagai & Thøgersen, 1987), and recombinant protein was purified essentially as described by Reinach and Karlsson (1988). Briefly, 4 × 1 L of 2 × TY broth (1.6% tryptone, 1.0% yeast extract, and 0.5% NaCl, pH 7.4), at 100 μg/mL ampicillin, was inoculated with pLcII-Fx-TnC-infected *E. coli* strain QY13, and the culture was incubated at 30 °C with shaking until a cell density of $A_{600} = 0.8$. Culture flasks were then transferred to a water bath at 60 °C and agitated until media temperatures were raised to 42 °C. The flasks were subsequently placed in an environmental shaker and incubated at 37 °C for 4 h.

After incubation, cultures were rapidly cooled on ice and cells harvested by centrifugation at 8000 rpm in a Sorvall GS3 centrifuge rotor at 4 °C. Cell pellets were resuspended in 110 mL of buffer A [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 8.0, 1 mM EDTA, 25% sucrose, and 0.5 mM PMSF]. Ten milliliters of a 24 mg/mL lysozyme solution was added to the suspension, and the mixture was incubated on ice for 30 min. After lysozyme treatment, 1.2 mL of a 1 mg/mL solution of DNase I, 2.4 mL of a 1.0 M solution of $MgCl_2$, and 120 μL of a 1.0 M solution of $MnCl_2$ were added to the suspension, and it was further incubated on ice for 30 min. After the addition of 0.6 mL of a 100 mM stock of PMSF in 2-propanol, the suspension was sonicated with three 20-s bursts at maximum power from a probe sonicator (Fisher Sonic-Dismembrator Model 300) fitted with a medium-sized probe. Cell debris was removed from the homogenate by centrifugation at 18 000 rpm in a Sorval SS34 rotor.

Ninety-six grams of urea, 31 mg of DTT, and 200 μL of a 1.0 M solution of Tris-HCl, pH 7.5, were added to the QY13 homogenate and dissolved. The entire mixture was loaded onto a 2.6 × 40 cm chromatography column packed with 160 mL of Q-Sepharose Fast Flow (Pharmacia) and preequilibrated in buffer B (50 mM Tris-HCl, pH 8.0, 8.0 M urea, 2 mM $MgCl_2$, 1 mM DTT, and 85 mM KCl). The column was washed with 500 mL of buffer B, and proteins were then eluted with a linear gradient from 85 mM KCl to 1.0 M KCl at a flow rate of 200 mL/h. Fusion troponin C was eluted at a salt concentration between 0.16 and 0.19 M KCl. Fusion troponin C was the most abundant protein in cell extracts and comigrated with known standards in SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

The column fractions from ion-exchange chromatography that contained fusion troponin C were pooled and desalted on a 2.6 cm × 90 cm chromatography column packed with 500 mL of G-25 Sephadex (Pharmacia). The column was preequilibrated in 0.1% TFA/H_2O and eluted with the same solvent at a flow rate of 85 mL/h. Fractions containing fusion protein were pooled, lyophilized, and stored desiccated at −20 °C. Typically, 200 mg of fusion protein was purified from 4 L of cell culture.

Factor Xa was prepared from bovine serum as described by Esnouf and Williams (1962). Conditions for the digestion of each preparation of fusion TnC by factor Xa were determined empirically. Typically, complete digestion was achieved in 12 h at room temperature in buffer C (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 2 mM $CaCl_2$) with an 800:1 w/w ratio of fusion protein to factor Xa.

Full-length TnC was purified from factor Xa digests by reversed-phase chromatography on a Spectra-Physics Model 8810 HPLC equipped with a 10 mm × 25 cm C4 (Vydac) column. The full-length protein was eluted from the column in a 20-min linear gradient extending from 65% solvent A (0.2% TFA/H_2O)/35% solvent B (0.2% TFA/acetonitrile) to 50% solvent A/50% solvent B. Purified recombinant TnC was lyophilized and stored at −20 °C until needed.

Mutagenesis. Oligonucleotides were purchased from the Regional DNA Synthesis Laboratory at the University of Calgary (Dr. R. Pons) and the Oligonucleotide Synthesis Laboratory at the University of British Columbia (Dr. T. Atkinson). The mutation phenylalanine-29 to tryptophan-29 (F29W) was directed by the 19-mer sequence 5′-T·GAC·ATG·TGG·GAT·GCG·GAC-3′, and the substitution phenylalanine-105 to tryptophan-105 was directed by the 17-

[1] Abbreviations: BSA, bovine serum albumin; DTT, DL-dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

mer sequence 5′-C·CGC·ATC·TGG·GAC·AAG·A-3′. Mismatches between the oligonucleotide and M13 template are underlined.

The coding region of chicken TnC had been cloned into the *Eco*RI site of bacteriophage M13mp19 (Messing, 1983). Site-specific mutagenesis was performed by the oligonucleotide-directed method of Zoller and Smith (1982) as described for double-priming by Carter et al. (1985). Mutant phage were sequenced in their entirety by the chain termination method (Sanger et al., 1977), using the enzyme Sequenase (version 2.0) and protocols provided by the U.S. Biochemical Corp. The DNA fragment bearing the substitution F29W was purified from replicative-form M13 by digestion with the restriction endonucleases *Sac*I and *Bst*XI. The fragment was subcloned into the vector pLcII-Fx-TnC (Reinach & Karlsson, 1988) which had been similarly cut with the enzymes *Sac*I and *Bst*XI. The recombinant mutant was designated pLcII-Fx-F29W.

Replicative-form M13 bearing the substitution F105W was digested with the enzymes *Sal*I and *Bst*XI. The appropriate fragment was then subcloned into vectors pLcII-Fx-TnC and pLcII-Fx-F29W to create the recombinant mutants pLcII-Fx-F105W and pLcII-Fx-F29W/F105W respectively.

Preparation of Samples for Analysis. Buffers prepared from reagent-grade water (16–18 MΩ, Milli-Q, Millipore Corp.) and filtered through chelating resin (Chelex, Sigma) were stored in acid-washed (6 N HCl) and rinsed plastic vessels.

Lyophilized protein samples, generally 5 mg, were dissolved in 1 mL of buffer D (50 mM MOPS, pH 6.95, 150 mM KCl, 1 mM EGTA, and 1 mM DTT) and dialyzed in plastic vessels for 30 h at 4 °C against two 500-mL changes of buffer D. Stock protein solutions were stored in liquid nitrogen until analysis. Protein concentrations were estimated by a modified version (Markwell et al., 1981) of the method of Lowry et al. (1951). (The Lowry method for estimation of recombinant troponin C, with BSA as a standard, was found to be in agreement with quantitation by amino acid analysis.)

For spectroscopic studies, stock solutions were diluted to the required concentrations with buffer D. All titrations were performed in thermostated cell assemblies at 25 ± 1 °C.

Fluorescence Spectroscopy. 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. Spectra and titrations were performed on samples (A_{280} < 0.05) in 1.0 cm² quartz cuvettes.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J-20 spectropolarimeter that was equipped with a thermostated cell assembly and interfaced to a microcomputer. Spectra were performed on samples in a quartz cuvette of 2.0-mm path length. The instrument was calibrated with D-pantolactone ($[\theta] = -16\,200$ deg·cm²·dmol⁻¹ at 219 nm, concentration = 0.015% w/v; Schippers & Dekkers, 1981).

UV Absorbance and UV Absorbance Difference Spectra. UV spectra were recorded on a Hewlett Packard Model 8452A diode array spectrophotometer with a 1-cm path length cell and 2.0-nm resolution. A split cell of 0.487-cm path length per chamber was used to generate absorbance difference spectra. Protein samples prepared in buffer D were placed in one chamber of the split cell, and calcium chloride solutions in buffer D were placed in the other chamber. An unmixed reference spectrum was recorded. Difference spectra were generated by subtracting the spectrum of the sample, after mixing of protein and calcium chloride solutions, from the spectrum of the unmixed blank.

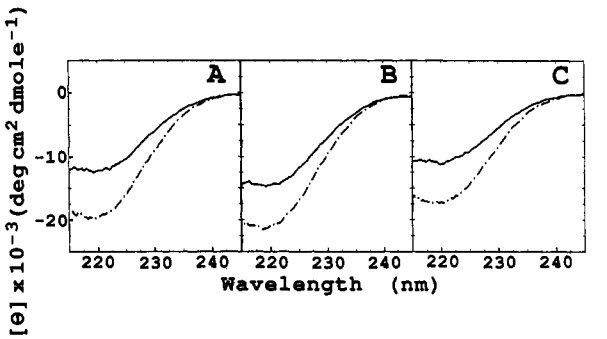

FIGURE 1: Far-UV circular dichroism spectra. Spectra were recorded of the recombinant proteins (A) 11.7 μM F29W, (B) 10.4 μM F105W, and (C) 10.1 μM F29W/F105W in buffer D, before and after the addition of calcium chloride to saturation (pCa < 4.0). The spectra of apoproteins appear as solid lines, and the spectra of proteins saturated with calcium appear as dashed lines.

Calculations. Free calcium concentrations were calculated from total calcium in chelating buffers by a computer algorithm described by Perrin and Sayce, (1967) and adapted to the MacIntosh microcomputer by Dr. B. Sykes and Mr. J. Boyko. Titration data were fitted by an iterative, derivative-free curve-fitting routine (BMDP Statistical Software, Inc.) on the IBM Model 3081 computer. The logarithmic binding constants for metals and H^+ to EGTA, used to calculate free ligand concentrations, were the following: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^{1-}, 2.0; Ca^{2+} to $EGTA^{4-}$, 10.72; Ca^{2+} to $HEGTA^{3-}$, 4.07; Mg^{2+} to $EGTA^{4-}$, 5.21; Mg^{2+} to $HEGTA^{3-}$, 3.37.

Atomic Absorbance Spectroscopy. Total calcium concentrations in calcium chloride stock solutions were determined by atomic absorbance on a Perkin-Elmer Model 1100B atomic absorption spectrophotometer. A 1.0 mg/mL calcium chloride standard (BDH) was used to calibrate measurements. All samples were prepared according to the protocol supplied by Perkin-Elmer (standard conditions for calcium).

RESULTS

After purification, the homogeneity of recombinant proteins was evaluated by SDS–polyacrylamide gel electrophoresis (data not shown). Each of the three purified proteins migrated as a single band on SDS gels. Recombinant mutants were analyzed by a combination of absorbance and fluorescence methods.

Far-UV Circular Dichroism. The far-UV CD spectra of recombinant mutants without and with calcium chloride at saturation (pCa = 4.0) appear in Figure 1. The corresponding ellipticity values and changes in ellipticity at 221 nm are presented in Table I. Calcium was observed to induce an increase in the magnitude of the negative ellipticities of F29W, F105W, and F29W/F105W. Although the absolute ellipticities of recombinant proteins varied, the relative changes in going from the calcium-free states of the proteins to calcium-saturated states were approximately equivalent and averaged -6.51×10^3 deg·cm²·dmol⁻¹.

Fluorescence. The fluorescence excitation spectra (not shown) of F29W, F105W, and F29W/F105W mutants, recorded at an emission wavelength of 335 nm, were characteristic of tryptophan and closely resembled the near-UV absorbance spectra of the proteins.

Fluorescence emission spectra of the recombinant proteins recorded before and after addition of calcium appear in Figure 2. The corresponding fluorescence quantum yields are

Table I: Relative Fluorescence Quantum Yields[a] and Far-UV Ellipticities[b] of Mutant Troponin C Proteins

mutant	Q_{rel}			$[\theta]_{221} \times 10^{-3}$ (deg·cm²·dmol⁻¹)		
	metal free	+Ca^{2+}	+Mg^{2+}	metal free	+Ca^{2+}	$\Delta\theta$
F29W	0.052	0.159		−11.76	−18.96	−7.20
F105W	0.066	0.054	0.104	−14.39	−20.52	−6.13
F29W/F105W	0.083	0.155		−10.63	−16.83	−6.20

[a] Fluorescence quantum yields are relative to free tryptophan $Q = 0.12$ (Borresen, 1967) dissolved in buffer D. Relative quantum yields were determined before, and after, the addition of metal ions (as $CaCl_2$ or $MgCl_2$) to a final free calcium concentration of 0.18 mM or a final free magnesium concentration of 89 mM. The excitation wavelength was 286 nm, and fluorescence emission was integrated between 295 and 420 nm. Protein concentrations and buffer conditions are identical to those in Figure 2. [b] Protein ellipticities were determined at 221 nm in the absence of calcium and after the addition of calcium to saturation (i.e., pCa < 4.0). Protein concentrations and buffer conditions are identical to those in Figure 1.

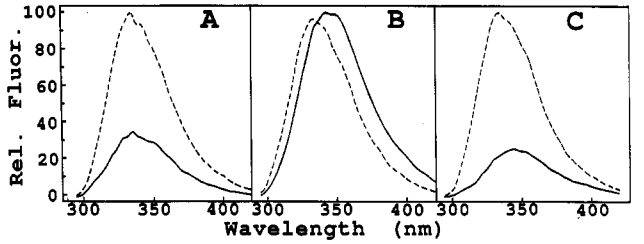

FIGURE 2: Fluorescence emission spectra. Fluorescence intensities (relative to maximal fluorescence) are graphed as a function of wavelength. The fluorescence emission spectra of the recombinant proteins (A) 5.0 μM F29W, (B) 5.0 μM F105W, and (C) 5.0 μM F29W/F105W in buffer D were recorded at an excitation wavelength of 278 nm, before (solid lines) and after (dashed lines) the addition of calcium chloride to a final free calcium concentration of 0.5 or 1.5 mM. Calcium saturation was determined by titration (Figure 3). The relative fluorescence quantum yields of calcium-free and saturated proteins are presented in Table I.

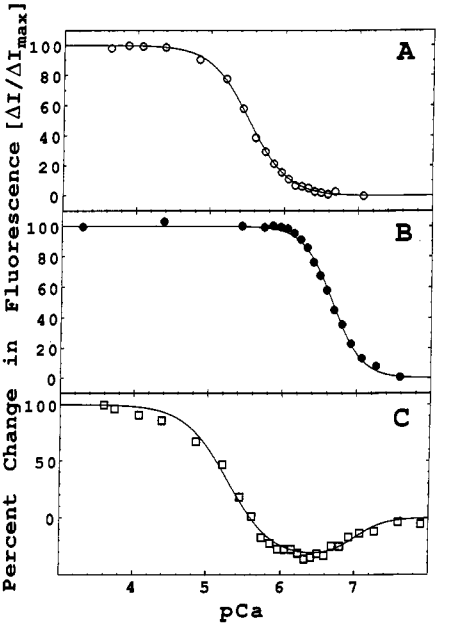

FIGURE 3: Titration of tryptophan fluorescence. The percentage change in fluorescence intensity was plotted as a function of the pCa. (A) F29W, (B) F105W, (C) F29W/F105W. Titration data are plotted as a percentage of the absolute change in the fluorescence intensity. Conditions are identical to those described in Figure 2, and binding parameters are presented in Table II. The fluorescence excitation wavelength was 278 nm (the excitation maxima) for all titrations, and emissions were monitored at 335 (F29W) or 366 nm (F105W and F29W/F105W).

presented in Table I. Calcium induced a 3.1-fold increase in the fluorescence quantum yield of the F29W protein. The F105W protein responded to calcium addition with a 1.22-fold reduction in the fluorescence quantum yield and a blue shift in the absorbance maximum from 344 nm (without Ca^{2+}) to 334 nm (with Ca^{2+}). The largest difference in the fluorescence intensity of the F105W apoprotein and Ca^{2+}-saturated F105W occurred at 366 nm, and calcium titrations were monitored at this wavelength. Calcium induced a 1.9-fold increase in the fluorescence quantum yield of F29W/F105W and a small blue shift in the emission spectrum from 336 to 333 nm. Calcium titrations of the F29W/F105W protein were monitored at an emission wavelength of 366 nm.

The effect of magnesium on the fluorescence emission spectrum of F105W is presented in Figure 5B, and the effect of magnesium on the fluorescence quantum yield is presented in Table I. Magnesium induced a 1.6-fold increase in the fluorescence quantum yield of F105W. Alternatively, magnesium had no influence on the fluorescence spectrum of the F29W protein.

Metal ion-induced changes in the fluorescence spectra of mutants allowed calcium binding to be titrated at low-affinity sites (Ca^{2+}-specific sites) and calcium and magnesium binding to be titrated at high-affinity (Ca^{2+}/Mg^{2+}) sites. The calcium titration curves of F29W, F105W, and F29W/F105W mutants appear in Figure 3, and binding data are presented in Table II. The fluorescence titration data of F29W and F105W were best fit by monophasic titration curves. The titration data of F29W/F105W were best fit by a binding equation that assumes there are two classes of binding sites. The magnesium titration curve of F105W appears in Figure 5C.

Absorbance Difference Spectra. The absorbance difference spectra of recombinant mutants perturbed with calcium appear in Figure 4. Difference spectra were generated by subtracting the absorbance spectrum of samples in the presence of metal ions from the absorbance spectrum of the same sample prior to the addition of metal ions. Difference spectra were generated for the mutant proteins at two free calcium concentrations, pCa = 6.1 and pCa = 3.7. According to the results of titration experiments (Figure 3), only high-affinity sites were expected to be occupied at a free calcium concentration of 0.9 μM, and all sites are expected to be occupied at a free calcium concentration of 0.18 mM. Significant changes were observed in the absorbance of F105W and F29W/F105W (Figure 4B,C, solid lines) with the first addition of calcium chloride (0.9 μM). Hence, the environment, or exposure, of tryptophan residues in these mutants is changed by calcium. No change in the environment of the tryptophan residue in the F29W mutant was apparent at the same concentration (Figure 4A). The second addition of calcium to a final free calcium concentration of 0.18 mM caused further transitions in the spectra and tryptophan environment of F29W and F29W/F105W, but no further change in F105W.

The absorbance difference spectrum of F105W in response to magnesium (Figure 5A) is similar to the absorbance difference spectrum of F29W in response to calcium (Figure 4A). In every case, absorbance difference spectra of mutant proteins were positive in response to calcium and magnesium

Table II: Binding Parameters Determined by Titration of Fluorescence[a]

mutant	calcium class	calcium K_{assoc} (M^{-1})	calcium h	magnesium class	magnesium K_{assoc} (M^{-1})	magnesium h
F29W	1 (low)	$(3.29 \pm 0.01) \times 10^5$	1.66 ± 0.03			
F105W	1 (high)	$(4.66 \pm 0.08) \times 10^6$	2.15 ± 0.07	1	$(4.64 \pm 0.01) \times 10^2$	1.44 ± 0.05
F29W/F105W	1 (high)	$(9.91 \pm 1.26) \times 10^6$	2.15 ± 0.53			
	2 (low)	$(1.90 \pm 0.10) \times 10^5$	1.47 ± 0.09			

[a] Titration data were fitted, as described under Materials and Methods, to an equation that assumes there is a single class of sites, $y = (K_1[Ca^{2+}])^h \cdot 100/[1 + (K_1[Ca^{2+}])^h]$, as in the case of F29W and F105W, or to an equation that assumes there are two classes of sites, $y = (K_1[Ca^{2+}])^{h_1}N_1/[1 + (K_1[Ca^{2+}])^{h_1}] + (K_2[Ca^{2+}])^{h_2}N_2/[1 + (K_2[Ca^{2+}])^{h_2}]$, as in the case of F29W/F105W. The percent saturation is given by y, K is an association constant, h is the Hill coefficient, and N is the percentage contribution to fluorescence. The subscripts 1 and 2 refer to the first and second class of sites, respectively. In fitting titrations of F29W/F105W, the best fit of binding data assumed that the parameters N_1 and N_2 were equal to −35 and 135, respectively.

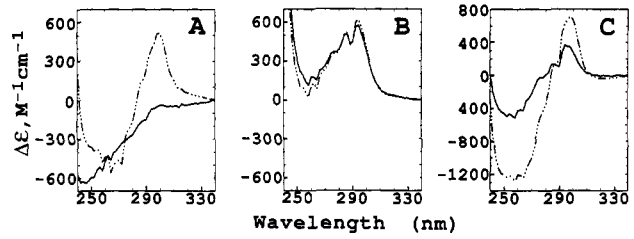

FIGURE 4: Absorbance difference spectra. Absorbance difference spectra were generated by subtracting the spectra of unmixed protein and calcium chloride solutions from the spectra of the same sample of protein and calcium chloride that had been mixed. (A) F29W, (B) F105W, (C) F29W/F105W. Solid lines represent the difference spectra of samples at a final protein concentration of 5 μM and a free calcium concentration of 0.9 μM. Dashed lines represent the difference spectra of samples at a final protein concentration of 5 μM and calcium at 0.18 mM.

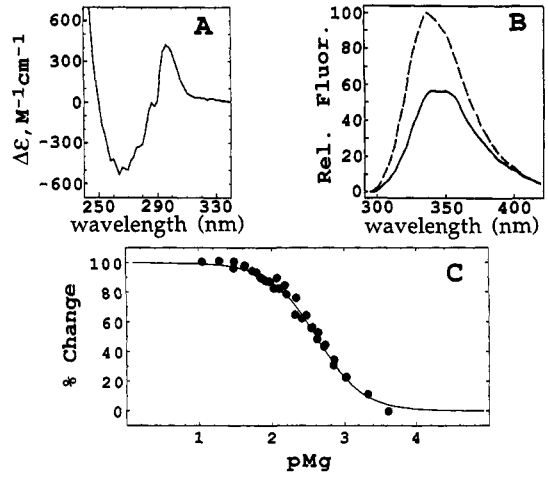

FIGURE 5: Spectroscopic properties of F105W in response to magnesium. (A) Absorbance difference spectrum of 5 μM F105W at a free magnesium concentration of 12.1 mM. Experimental conditions were as described in Figure 4. (B) Fluorescence emission spectra of 5 μM F105W before (solid line) and after (dashed line) titration to a final free magnesium concentration of 89 mM. Experimental conditions were as described in Figure 2. (C) Titration of the magnesium-induced change in tryptophan fluorescence of F105W. The excitation wavelength was 278 nm, and emissions were recorded at 345 nm. Other experimental conditions were as described in Figure 3.

ions. Therefore, the absorbance spectra of the substituted tryptophans are red-shifted by metal ions.

DISCUSSION

Tyrosine and tryptophan are unique in comparison with other amino acids. They have absorbance maxima near 280 nm, high extinction coefficients, and high fluorescence quantum yields (Cantor & Schimmel, 1980). As a consequence, these amino acids are well suited to function as site-specific probes, or reporters, of ligand binding and conformational change in proteins (Chen et al., 1969). Chicken troponin C does not naturally contain either tyrosine or tryptophan (Wilkinson, 1976). Therefore, it was possible for us to assign fluorescence and absorbance properties of mutant troponin C's unambiguously to the positions of tryptophan substitution.

The published three-dimensional structures of chicken TnC (Satyshur et al., 1988) and turkey TnC (Herzberg et al., 1987) show a protein that is organized into two domains separated by a long interdomain α-helix. Binding sites have a helix–loop–helix structural motif (Kretsinger & Nockolds, 1973) containing an arrangement of six residues which are involved, either directly or indirectly, in coordinating metal ions (Strynadka & James, 1989). Coordination takes place through interactions with the oxygen atoms of (a) main-chain carbonyls, (b) side-chain carboxylates, and (c) water. Only high-affinity sites are seen to be occupied by metal ions in the structures of Herzberg and James (1985) and Satyshur et al. (1988).

It is now generally accepted that metal ion binding is associated with a significant reorganization of structure in the binding domains (Strynadka & James, 1989; Grabarek et al., 1990; Fujimori et al., 1990; Pearlstone et al., 1992) and that changes in conformation are responsible for transmitting signals for fiber contraction to other proteins in the troponin complex, and beyond. Herzberg et al. (1986) have proposed a model for the structural reorganization of low-affinity sites on binding calcium. The basis for this model is the structural homology, seen in crystal diffraction studies, of the "occupied" high-affinity sites with the "unoccupied" low-affinity sites. In essence, the crystal structure of high-affinity sites becomes a model for the structure of low-affinity sites with metal ions bound. To date, no X-ray crystal structure of TnC has appeared in which low-affinity sites are "occupied".

The phenylalanine which naturally resides at position 29 of chicken TnC precedes the first coordinating residue of binding loop I in the low-affinity domain. In the high-affinity domain, phenylalanine-105 occupies a position analogous to Phe-29 and precedes the first coordinating residue of binding loop III. Because of the close proximity of tryptophans in the TnC mutants to binding loops (Herzberg & James, 1985; Herzberg et al., 1986), spectral properties of mutant proteins were predicted to be strongly influenced by metal ion binding and conformational change.

Far-UV Circular Dichroism. The integrity of mutant proteins was evaluated by far-UV circular dichroism (Figure 1). Calcium has been reported to cause an increase in the magnitude of the negative ellipticity of troponin C at 221 nm (Hincke et al., 1978; Johnson & Potter, 1978), corresponding to an increase in the helical content of the protein. Between 60% and 70% of the contribution to negative ellipticity was

associated with high-affinity metal ion binding and the remaining contribution associated with low-affinity transitions. The changes in ellipticity observed in tryptophan mutants (Table I) are comparable, within the error of the method, to those values reported for rabbit skeletal TnC. For example, Hincke et al. (1978) reported that calcium induced an absolute change in protein ellipticity of 8.86×10^3 deg·cm^2·dmol^{-1} at pH 6.94 and the average change in the ellipticity of recombinant mutants was 6.51×10^3 deg·cm^2·dmol^{-1} at pH 6.95.

As reported in studies of rabbit skeletal TnC, changes in the far-UV circular dichroism of the mutant proteins are dominated by high-affinity transitions. Low- and high-affinity transitions were found to overlap (far-UV CD titration data not shown). Far-UV CD studies suggest that the overall folding and function of mutant proteins are not significantly altered by tryptophan substitutions. The calcium-induced changes in the CD spectra of the three mutant proteins were similar to one another, regardless of the location of tryptophans in the high-affinity domain (F105W), low-affinity domain (F29W), or both domains (F29W/F105W).

Fluorescence. Fluorescence was the most sensitive and reproducible method of monitoring metal ion binding (Figure 2). The calcium-induced increase in the fluorescence intensity of the F29W protein permitted low-affinity calcium binding sites to be titrated at 335 nm (Figure 3A). The calcium-induced decrease in the fluorescence intensity of the F105W protein permitted us to titrate high-affinity calcium binding sites at 366 nm (Figure 3B). These titrations gave estimates of $K_a = (3.29 \pm 0.01) \times 10^5$ M^{-1} and Hill coefficient = 1.66 ± 0.03 for low-affinity binding and $K_a = (4.66 \pm 0.08) \times 10^6$ M^{-1} and Hill coefficient = 2.15 ± 0.07 for high-affinity calcium binding to recombinant chicken troponin C. The simultaneous titration of two tryptophans in the F29W/F105W double mutant was observed at 366 nm (Figure 3C). Since titration curves overlap and since the contributions of the two tryptophans in F29W/F105W to total fluorescence are not equivalent, it was not possible to estimate binding constants with the same precision as in the single mutants. Similar problems were encountered when the binding constants were estimated by titration of the far-UV CD (data not shown).

Magnesium had no significant effect on the fluorescence emission (or absorbance properties) of the F29W protein and, as reported elsewhere (Potter & Gergely, 1975; Pearlstone et al., 1992), does not appear to bind to the so-called Ca^{2+}-specific sites. However, at saturation, magnesium had a significant effect on the fluorescence emission of the F105W protein, bringing about a 1.6-fold increase in the fluorescence quantum yield. The increase in the fluorescence quantum yield allowed us to titrate magnesium binding at 345 nm (Figure 5C).

Absorbance Spectra. The absorbance difference spectra provided an indication of the type of change in environment tryptophans experience when metal ions are bound. A positive change in the extinction coefficient is observed in the difference spectra (Figure 4) from approximately 275 to 320 nm. Therefore, the absorbance spectrum of tryptophan at either position 29 or position 105 is red-shifted by calcium ions. Magnesium ions also red-shift the absorbance spectrum of F105W. It is interesting to note the similarity of spectra in Figures 4A and 5A. The tryptophan in the high-affinity domain (F105W) undergoes a magnesium-induced change in environment that is virtually identical to the calcium-induced change in the environment of the tryptophan in the low-affinity domain (F29W). It is also worth noting (though fluorescence decay mechanisms have not been established) that magnesium induces an increase in the fluorescence quantum yield of F105W similar to the increase induced by calcium in F29W (Table I).

At high concentrations of magnesium, such as those used to titrate Ca^{2+}/Mg^{2+} sites in F105W, calcium may become a significant contaminant. Therefore, it is questionable whether calcium or magnesium is titrating the sites. By comparing the absorbance difference spectra for F105W in Figures 4B and 5A, it is clear that calcium and magnesium ions have quite different effects on the environment of the tryptophan chromophore. Therefore, the magnesium titration of F105W is a true estimate of the magnesium binding constant. It is not surprising that calcium and magnesium have different effects on the absorbance and fluorescence properties of tryptophan chromophores. The ionic radii of calcium and magnesium are 0.99 and 0.64 Å, respectively. Therefore, the conformation of the high-affinity Ca^{2+}/Mg^{2+} sites that best accommodates calcium is not optimal for the coordination of magnesium.

Given that the tryptophan probes are engineered into homologous positions in the high- and low-affinity domains, it appears at first surprising that calcium-induced changes in the spectral properties of Trp105 are different from the calcium-induced changes in Trp29. Yet, it can be seen by the comparison of calcium-induced and magnesium-induced changes in the F105W mutant that only very subtle changes in structure (i.e., the difference in the dimensions of the two ions) are required to bring about large changes in the spectral properties of the reporter group. Accordingly, the difference in the response of Trp105 and Trp29 to calcium merely reflects subtle differences in the structures of the two domains.

According to the conformational change model proposed by Herzberg et al. (1986), a phenylalanine residue at position 29 will experience a change in surface exposure from 46.2 to 55.7 Å^2, an increase of 9.5 Å^2. The red shift in the absorbance spectra of the F29W and F105W proteins observed in Figure 4A,B is consistent with the tryptophan becoming more buried or less exposed. There are several possible explanations for the difference between the observed and predicted transitions of a side chain at position 29. For example, the tryptophan in this position may adopt a conformation somewhat different than phenylalanine, or the conformational change model may not be entirely accurate.

As shown in this study, and discussed at length in Pearlstone et al. (1992), high cooperativity is a feature of metal ion binding at both Ca^{2+}-specific and Ca^{2+}/Mg^{2+} sites. Yet, there is contradictory evidence of cooperativity in the many previous reports of skeletal troponin C. The studies described here, in Golosinska et al. (1991), and in Pearlstone et al. (1992) suggest that the methods used to monitor metal binding, as well as the conditions under which proteins are prepared, can influence the observation of cooperativity. The use of bulky, and charged, extrinsic probes may be particularly disruptive to troponin C.

The binding constants for calcium and magnesium at high-affinity sites in recombinant troponin C are lower than those previously reported for nonrecombinant rabbit skeletal troponin C [K_a values of 2.1×10^7 and 4.0×10^3 M^{-1} for calcium and magnesium, respectively (Potter & Johnson, 1982)]. This difference has been correlated with the variation of amino acid residues at position 130 in the high-affinity domain and is discussed in detail by Golosinska et al. (1991). In rabbit skeletal troponin C, as well as in native chicken TnC, position 130 is occupied by a threonine residue. The same position of the recombinant chicken TnC isolated by Reinach and Karls-

son (1988) is occupied by isoleucine. We are currently employing the mutant F105W in a study which explores the influence of amino acid substitutions at position 130 on helix stability, metal ion binding, and specificity.

In summary, we have demonstrated that site-specific mutagenesis is a facile mechanism for the introduction of intrinsic reporters into recombinant troponin C. The tryptophan substitutions at positions 29 and 105 allowed binding events to be monitored in one domain of the protein independently of binding events in the other domain. Being able to independently monitor binding at the two classes of sites may prove to be important in future mutagenesis studies where high-affinity and low-affinity titration curves overlap severely (as is the case in far-UV CD titrations). Titration data from the single-tryptophan mutants can be fit to equations requiring only two parameters, namely, a single binding constant and a single Hill coefficient. Fitting data from the simultaneous titration of two classes of sites by titration of, for example, far-UV ellipticity involves six parameters: two binding constants, two Hill coefficients, and the relative contribution of each class of site to the spectral feature. The results would, consequently, have less statistical significance. In addition to the aforementioned benefits of the tryptophan mutants, F105W showed that calcium and magnesium ions induce significantly different conformational transitions in the high-affinity domain of troponin C, an observation that may have implication to the function of high-affinity sites in the troponin complex. In conclusion, the tryptophan-substituted proteins described here represent a reference point for further structure/function studies by mutagenesis.

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