Free-Energy Linkage between Folding and Calcium Binding in EF-Hand Proteins

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ABSTRACT   Troponin is the singular Ca^{2+}-sensitive protein in the contraction of vertebrate striated muscles. Troponin C (TnC), the Ca^{2+}-binding subunit of the troponin complex, has two distinct domains, C and N, which have different properties despite their extensive structural homology. In this work, we analyzed the thermodynamic stability of the isolated N-domain of TnC using a fluorescent mutant with Phe 29 replaced by Trp (F29W/N-domain, residues 1–90). The complete unfolding of the N-domain of TnC in the absence or presence of Ca^{2+} was achieved by combining high hydrostatic pressure and urea, a maneuver that allowed us to calculate the thermodynamic parameters (ΔV and ΔG_{atm}). In this study, we propose that part of the affinity for Ca^{2+} is contributed by the free-energy change of folding of the N- and C-domains that takes place when Ca^{2+} binds. The importance of the free-energy change for the structural and regulatory functions of the TnC isolated domains was evaluated. Our results shed light on how the coupling between folding and ion binding contributes to the fine adjustment of the affinity for Ca^{2+} in EF-hand proteins, which is crucial to function.

INTRODUCTION

Troponin C (TnC) is a member of the EF-hand family, which encompasses several proteins sharing a common helix-loop-helix motif responsible for Ca^{2+} binding (1,2). TnC is a subunit of the troponin complex (TnC, TnI, and TnT), which, in concert with tropomyosin, modulates the cyclic interaction between myosin and actin during muscle contraction (for review, see the articles by Farah and Reinach (3); Tobacman (4); Squire and Morris (5); and Gordon et al. (6)).

The Ca^{2+}-dependent competition between actin and TnC for the inhibitory peptide region of TnI is a key feature of current models of the mechanism of Ca^{2+} signaling in the regulated thin filament. NMR structural studies have demonstrated a bulk movement of helices B and C of the N-domain with little change in helical content on Ca^{2+} binding (7). This change in conformation exposes a hydrophobic pocket that is part of an antiparallel motif for the interaction with TnI and initiates signal transmission to the rest of the regulated thin filament (3,8). These structural alterations are referred to as the opening of the N-domain. Thus, the N-domain of TnC plays a crucial role in muscle contraction.

Sites I and II located in the N-domain bind Ca^{2+} specifically with a relatively low affinity ($K_{a[Ca]} \sim 10^5 M^{-1}$), whereas sites III and IV in the C-domain bind Ca^{2+} with a higher affinity ($K_{a[Ca]} \sim 10^7 M^{-1}$) and also bind Mg^{2+} ($K_{a[Mg]} \sim 10^3 M^{-1}$)

(9). In addition to the two classes of sites, there is evidence for an additional class, the weak sites, that bind Mg^{2+} and Ca^{2+} with very low affinity ($K_{a[Ca,Mg]} \sim 10^2$–$10^3 M^{-1}$) (9–13). Recently, we observed that Ca^{2+} and Mg^{2+} binding to weak sites of the TnC C-domain induces exposure of a large hydrophobic surface that leads to loss of TnC from the thin filament (13). It is postulated that the C-domain has a structural role, whereas the N-domain displays regulatory function for the reasons stated above.

In a previous study (14), we used F105W/C-domain (residues 88–162) and F105W (residues 1–162) to address questions related to the stability of the C-domain, using W105 (located in site III) as a probe. Monitored by changes in Trp emission, the C-domain apo state was denatured by pressure (in the range of 1–1000 bar) in the absence of urea. The addition of Ca^{2+} or Mg^{2+} increased the C-domain stability so that complete denaturation was attained only by the combined use of high hydrostatic pressure and subdenaturing concentrations of urea. This combination allowed us to calculate the thermodynamic parameters (ΔV and ΔG_{atm}°) for folding of the C-domain in the presence of Ca^{2+} or Mg^{2+}. In this study, we used the F29W/N-domain (residues 1–90) to address questions related to the stability of the N-domain after the spectroscopic changes that take place in the N-domain using W29, close to the beginning of the Ca^{2+}-binding loop of site I. Upon Ca^{2+} binding, the fluorescence emission of W29 increases ~3-fold, suggesting that this residue reflects the loss of a nearby quenching group. We have shown previously (15) that the combination of high pressure and low temperature ($-11°C$) in the absence of added Ca^{2+} promotes the same increment in the fluorescence emission of the F29W/N-domain. This finding suggests that pressure and low temperature together can drive the N-domain of TnC into a conformation that resembles the conformation caused by Ca^{2+} binding. The explanation for this observation was that low

temperatures, in combination with high pressure, weaken hydrophobic interactions, thus facilitating the opening of the N-domain. The possibility of trapping this opened conformation under pressure enabled us to calculate the thermodynamic parameters associated with the opening of the N-domain. The free-energy change found was small and positive (1.9 kcal/mol). Under physiological conditions, the binding of Ca^{2+} to the N-domain overcomes this barrier and displaces the equilibrium toward exposure of the hydrophobic patch that anchors TnI so that the overall reaction occurs spontaneously.

In this study, we investigate the thermodynamic stability of the F29W/N-domain by combining high pressure with subdenaturing concentrations of urea. High pressure has been a crucial tool in exploring the thermodynamics and kinetics of protein folding and protein interactions (16–21). Using this maneuver, we constructed a complete thermodynamic cycle for the F29W/N-domain in all of its occupancy states. Additionally, by performing pressure titration at 1°C, we evaluated the effects of low temperature on the thermodynamic stability of the F29W/N-domain, because the N-domain has a higher affinity for Ca^{2+} in this condition (15). In combination with our previous article (14) in which denaturation of the C-domain was investigated, we observed that the increase in stability of the C-domain when Ca^{2+} binds is >50% greater than that caused by Ca^{2+} binding to the N-domain. However, the sites in the C-domain (III and IV) have a higher affinity for Ca^{2+} than the sites in the N-domain (I and II). We propose that part of this higher affinity for Ca^{2+} comes from the greater free-energy change of folding that takes place when Ca^{2+} binds to the C-domain. Our data illustrate the importance of free-energy linkage between folding and calcium binding in EF-hand proteins. The physiological relevance of the thermodynamic parameters for muscle contraction is also discussed.

MATERIALS AND METHODS

Reagents and protein preparation

The N-domain mutant (F29W/N-domain, residues 1–90) was prepared as described previously (22). All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore (Billerica, MA) water purification system. The experiments under pressure were performed in the standard buffer (100 mM Tris-HCl, 100 mM KCl, 1.0 mM diothiothreitol (DTT), and 1.5 mM EGTA, pH 7.0). Tris-HCl buffer was selected as a buffer because the dependence of its pK_a on pressure is small. At 3.0 kbar, the pK_a increases by only 0.1 units (23). Temperature control was achieved through the use of a circulating water bath.

Spectroscopic measurements

The Trp fluorescence spectra were recorded on a spectrofluorometer (ISSK2; ISS, Champaign, IL). Samples were excited at 280 nm, and the emission was collected from 300 to 400 nm. Tryptophan fluorescence spectra at pressure p or in the presence of any concentration of urea u were quantified as the center of spectral mass $\langle \nu_{p,u} \rangle$, in cm^{-1} as follows:

$$\langle \nu_{p,u} \rangle = \Sigma \nu_i F i / \Sigma F i, \qquad (1)$$

where Fi stands for the fluorescence emitted at wavenumber ν_i and the summation is carried out over the range of appreciable values of F.

The degree of denaturation (α) in a given condition was calculated from the changes in center of spectral mass (ν) in that condition as follows:

$$\alpha = (\langle \nu \rangle_i - \langle \nu \rangle)/(\langle \nu \rangle_i - \langle \nu \rangle_f), \qquad (2)$$

where $\langle \nu \rangle_i$ is the initial value of center of spectral mass (native protein) and $\langle \nu \rangle_f$ is the final value (unfolded protein). Spectra were recorded after 5 min of equilibration at each pressure. For the urea denaturation curves, the samples were incubated for 1 h in each concentration of urea. The protein concentration was 5 μM in all measurements. All spectroscopic changes reported were completely reversible.

Thermodynamic parameters

The Gibbs free-energy change and the equilibrium constant for a given reaction depend on the standard volume change of the reaction (ΔV) according to the relation as follows:

$$K_f(p) = K_{atm} \exp(p\Delta V/RT), \qquad (3)$$

where $K_f(p)$ and K_{atm} are the equilibrium constants of folding at pressure p and 1 bar, respectively. If we introduce the extent of reaction at pressure p, α_p, we deduce the following thermodynamic relation (24,25) as follows:

$$\ln [\alpha_p/(1 - \alpha_p)] = p(\Delta V/RT) + \ln K_{atm}. \qquad (4)$$

For apo and Ca^{2+}-bound states, pressure alone was not enough to promote a complete unfolding. Then, the experiments were performed in the presence of increasing concentrations of urea. However, the volume change (ΔV) increased linearly with the addition of urea, leveling off at the highest concentration of urea used (Table 1). This type of behavior makes the use of the direct relation between ΔG and the unfolding constant (K_{atm}) inappropriate. Thus, we first determined the free-energy change at 1241 bar in the presence of different concentrations of urea (Eq. 5). The extrapolation of this plot to 0 M urea gives ΔG^{o0M}_{1241}. This value was introduced into Eq. 6 to determine the free-energy change at atmospheric pressure (ΔG^{o}_{atm}) as follows:

$$\Delta G^{o[U]}_{1241} = m[U] + \Delta G^{o0M}_{1241} \qquad (5)$$

$$\Delta G^{o0M}_{1241} = \Delta G^{o}_{atm} + p\Delta V, \qquad (6)$$

where $\Delta G^{o[U]}_{1241}$ and ΔG^{o0M}_{1241} represent the free-energy change at 1241 bar in the presence or absence of urea, respectively, and m is a constant proportional to the difference in solvent-accessible surface area as the protein unfolds (26). The volume change used in Eq. 6 was the highest value observed (marked in bold in Table 1). We chose 1241 bar because this pressure is close to the p_{50} values of the majority of the curves. In all experiments, the spectroscopic changes were reversible after pressure release.

Circular dichroism measurements

Circular dichroism (CD) measurements were performed in a spectropolarimeter (Jasco-715; Jasco, Tokyo, Japan) using a 1.0 mm pathlength quartz cuvette. For spectra determinations, stock solutions of protein were prepared in 10 mM Tris-HCl, 100 mM KCl, 1 mM DTT, and 1.5 mM EGTA, at pH 7.0, in the absence or in the presence of 2.1 mM $CaCl_2$ and 8 M urea. Data were averaged for two scans at a speed of 50 nm/min collected in 0.2 nm steps.

RESULTS

Spectroscopic changes of the F29W/N-domain in the apo and Ca²⁺-bound states during unfolding

The Trp in the N-domain presents a threefold increase in its emission (Fig. 1) and a slight blue shift (152 cm^{-1}) when the apo protein binds Ca^{2+} (Fig. 1, *inset*). This displacement of the spectrum to the blue reflects the movement of Trp to the hydrophobic core of the protein. As expected (22), the addition of Mg^{2+} did not alter the fluorescence spectrum of the F29W/N-domain (data not shown).

The spectroscopic changes that take place during urea-induced unfolding of the F29W/N-domain in the apo and Ca^{2+}-bound states at 20°C are depicted in Fig. 2. In the absence of divalent cations, the center of mass in 8 M urea lies at $28,414 \text{ cm}^{-1}$. This value reflects the exposure of the Trp to the aqueous environment, suggesting that urea promotes complete denaturation of the F29W/N-domain. Strikingly, the center of spectral mass of the Ca^{2+}-bound form did not change even at high concentrations of urea. These data show that the structure of the F29W/N-domain is remarkably stable when Ca^{2+} is bound, as seen with the C-domain (14). The unfolding process is better visualized when the degree of denaturation (α) is plotted as a function of urea concentration (Fig. 2, *inset*).

The structural stability of the N-domain was also analyzed by CD experiments. Fig. 3 shows the far-ultraviolet (UV) CD spectra of the F29W/N-domain in the absence and presence of Ca^{2+} with or without 8 M urea. The addition of Ca^{2+} induced an increase in negative ellipticity that can be attributed, in part, to an increase in its α-helical content (22). In agreement with the result obtained by fluorescence (Fig. 2), Ca^{2+} binding to
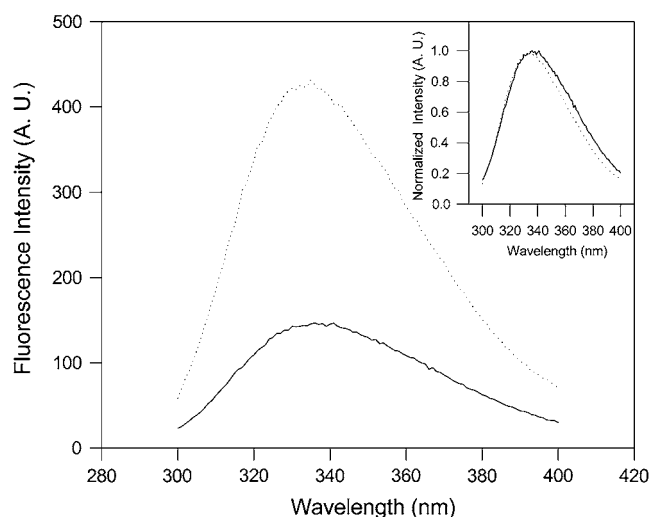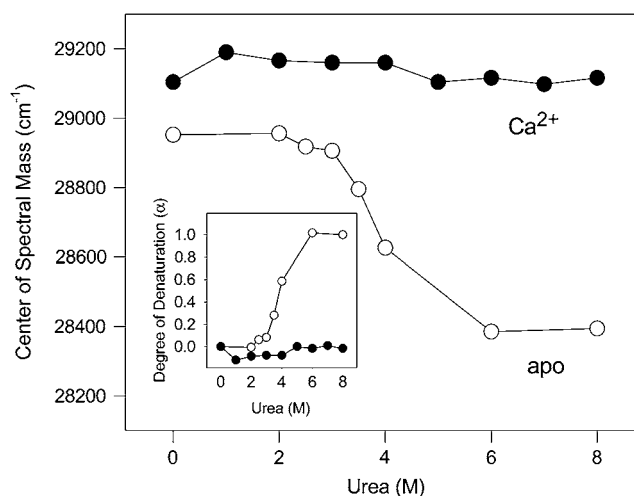
FIGURE 2 Exposure of W29 after the addition of urea. The F29W/N-domain in the absence (*open circles*) or presence (*solid circles*) of 2.1 mM CaCl₂. (*Inset*) Extent of unfolding (α) for the urea-induced unfolding of the F29W/N-domain as calculated in Materials and Methods. Other conditions are the same as those described in Fig. 1 legend.

the F29W/N-domain stabilized the secondary structure, because 8 M urea produced no change in the CD signal at 222 nm. In contrast, the apo form of the F29W/N-domain was completely denatured in the presence of 8 M urea. The profile of urea denaturation, as measured by the changes in CD, overlaps with those obtained by the changes in fluorescence spectra (data not shown), indicating that the exposure of the W29 residue to the aqueous environment reflects the changes in global folding rather than only local changes.

Pressure-induced denaturation of the F29W/N- domain

To study the unfolding of the F29W/N-domain in the presence of Ca^{2+}, we took advantage of the combined use of high pressure and subdenaturing concentrations of urea as perturbing agents. Figs. 4 and 5 show the effects of high pressure on the structure of the F29W/N-domain in the apo and Ca^{2+}-bound states at 20°C (Fig. 4) and 1°C (Fig. 5). High pressure in the absence of urea did not lead to complete exposure of Trp to the solvent, irrespective of whether Ca^{2+} was present (Fig. 4, *open circles*). However, by combining pressure and subdenaturing concentrations of urea, it was possible to observe a large red shift in the Trp emission to a value compatible with its complete exposure to the aqueous environment ($\sim28,400 \text{ cm}^{-1}$). As expected, the concentrations of urea necessary to denature the N-domain in the apo state were lower (2.5–3.5 M) than in the Ca^{2+}-bound state (6.0–8.0 M).

Thermodynamic parameters of folding

From the pressure-denaturation curves in the presence of increasing concentrations of urea, it was possible to calculate the

FIGURE 1 Fluorescence emission spectra of the F29W/N-domain in the absence (*solid line*) or presence of 2.1 mM CaCl₂ (*dotted line*). (*Inset*) Normalized fluorescence emission spectra. The protein concentration was 5 μM. The samples were excited at 280 nm, and the emission measured from 300 to 400 nm. The solvent conditions were 100 mM Tris-HCl, 100 mM KCl, 1.0 mM DTT, and 1.5 mM EGTA, pH 7.0, at 20°C.
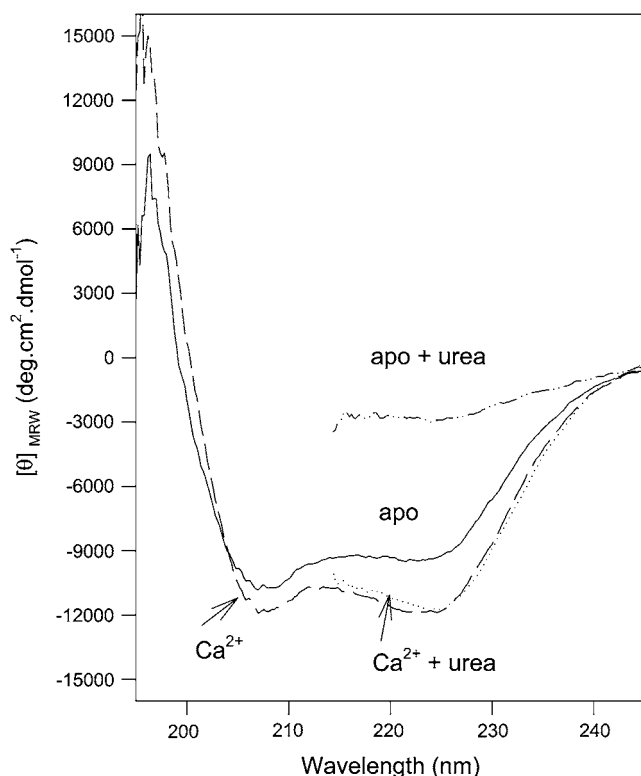
FIGURE 3 Far-UV CD spectra of the F29W/N-domain. Conditions: apo form (*solid line*); plus 2.1 mM CaCl$_2$ (*dashed line*); apo form in the presence of 8 M urea (*dotted/dashed line*); and plus 2.1 mM CaCl$_2$ in the presence of 8 M urea (*dotted line*). Protein concentration was 12 μM. The solvent conditions were 10 mM Tris-HCl, 100 mM KCl, 1.0 mM DTT, and 1.5 mM EGTA, pH 7.0.

volume change of folding of the F29W/N-domain in the apo or Ca^{2+}-bound state by the use of Eq. 4. Values of volume change (ΔV) of folding obtained at 20°C and 1°C are shown in Table 1. As seen with the C-domain, the volume change of folding increased with urea concentration until saturation was attained. The highest ΔV values were assumed to represent the equilibrium values. At 20°C, the highest values were 48.6 mL/mol and 60.7 mL/mol, which were obtained in the absence and presence of Ca^{2+}, respectively. As observed in Table 1, lowering the temperature did not affect the maximal ΔV.

Table 2 shows the changes in volume derived from the pressure effects on the N-domain structure as evaluated by ^{1}H-NMR spectroscopy. Because the high-pressure NMR cell can withstand up to 5 kbar (27), it is possible to derive the values of volume change in the absence of urea for the conditions without Ca^{2+}. As observed for the C-domain of TnC (14), the largest magnitudes for ΔV were associated with the amino acid residues that are located in the hydrophobic core of the protein. The deviations in ΔV along the protein demonstrate how unfolding affects protein structure in a site-specific manner, which noticeably depends on the stabilizing forces in the local environment of each residue. The values obtained by NMR for the amino acid sites located in the hydrophobic core
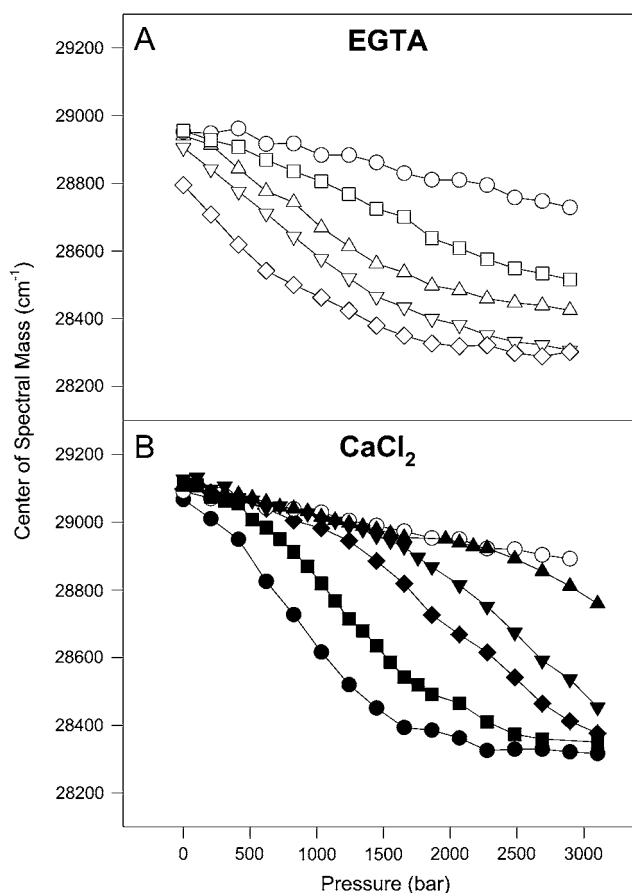

FIGURE 4 Effects of high pressure combined with urea on the center of spectral mass of the F29W/N-domain, at 20°C. (*A*) In the absence of divalent cations: without urea (*open circles*); plus 2 M (*open squares*), 2.5 M (*open triangles facing up*), 3.0 M (*open triangles facing down*), or 3.5 M (*open diamonds*) urea; and (*B*) in the presence of 2.1 mM CaCl$_2$: without urea (*open circles*); plus 4 M (*solid triangles up*), 5 M (*solid triangles down*), 6 M (*solid diamonds*), 7 M (*solid squares*), or 8 M urea (*solid circles*). Protein concentration was 5 μM. All other conditions were the same as those described in Fig. 1 legend.

in the absence of urea (30–42 mL/mol) are close to those obtained using the changes in the fluorescence of W29 in the presence of urea (Table 1). The tryptophan fluorescence reflects the overall tertiary structure, whereas the NMR reflects the changes in the local environment of the amino acid residues. However, as observed for troponin C C-domain (14), the close correspondence between the volume change obtained by fluorescence and those values measured by NMR for residues that are closely packed in the hydrophobic core permits us to use fluorescence to explore the pressure denaturation of the N-domain at different concentrations of urea.

To obtain the ΔG°_{atm} for the folding process at 20°C and 1°C from the fluorescence experiments, we calculated the free-energy change at 1241 bar ($\Delta G^{\circ 0M}_{1241}$). This approach was used because of the two slopes present in the unfolding curves in the presence of Ca^{2+}. Thus, we calculated the free-energy change at 1241 bar in the presence of different concentrations of urea for the apo or Ca^{2+}-bound state of the F29W/N-domain (Fig. 6).
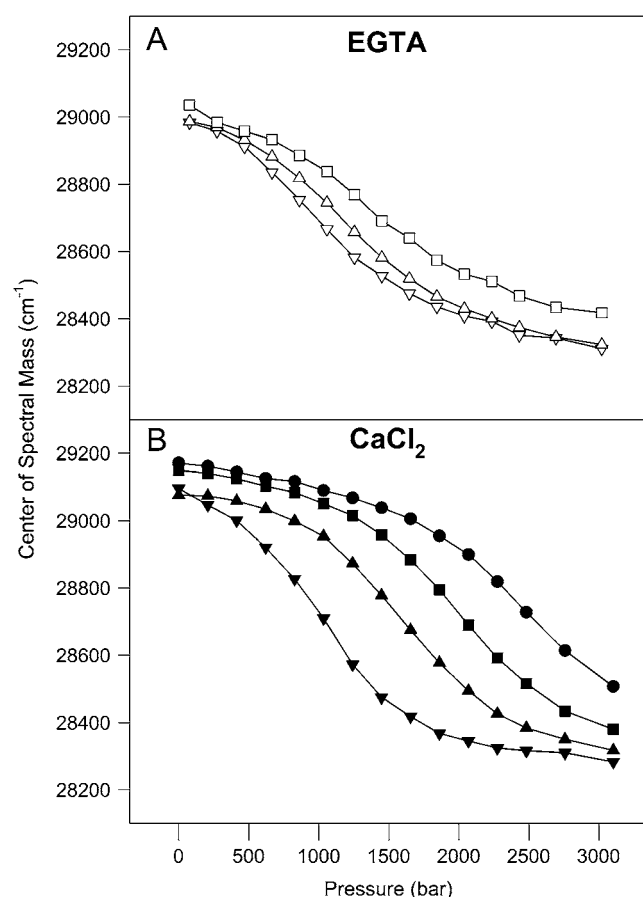
FIGURE 5 Effects of high pressure combined with urea on the center of spectral mass of the F29W/N-domain at 1°C. (*A*) In the absence of divalent cations: plus 2.5 M (*open squares*); 3.0 M (*open triangles up*), or 3.5 M (*open triangles down*) urea; and (*B*) in the presence of 2.1 mM CaCl₂: plus 5.0 M (*solid circles*); 5.5 M (*solid squares*); 6.0 M (*solid triangles up*) or 7.0 M (*solid triangles down*) urea. Protein concentration was 5 μM. All other conditions were the same as described in Fig. 1 legend.

The extrapolation of these curves to the *y* axis (0 M urea) furnishes $\Delta G°$ in the absence of urea at 1241 bar ($\Delta G°^{0M}_{1241}$). Through the use of Eq. 6, we assessed the $\Delta G°$ at atmospheric pressure in the absence of urea ($\Delta G°_{atm}$). For the F29W/N-domain apo form at 20°C and 1°C, these values were −3.4 and −3.1 kcal/mol, respectively. In the Ca²⁺-bound form, the

$\Delta G°_{atm}$ of folding was −5.8 at 20°C and −6.8 kcal/mol at 1°C (Table 3).

DISCUSSION

TnC is the Ca²⁺-binding subunit of the troponin complex and has two distinct domains, C and N, which show different properties despite their high structural homology. In this study, we analyzed the thermodynamic stability of the N-domain of TnC using a fluorescent mutant with Phe 29 replaced by Trp. In a previous study (27), we used the atomic structures available for TnC (28,29) to perform molecular simulations on the replacement of phenylalanine at position 29 with tryptophan. Energy-minimized structures of wild-type and F29W chicken skeletal N-domain fragments (residues 3–90) were obtained from the 4tnc x-ray structure (28) and showed that the mutation minimally disrupts the main chain carbons of the protein. The calcium-binding loop I, adjacent to the mutation, showed a small change in structure because of the adjustment to W29, a larger and less hydrophobic residue. However, the molecular simulations clearly demonstrated that there are only minor changes in the position of the side chains in the hydrophobic core of the mutant form compared to the wild-type. In contrast, the localization of W29 in a hydrophobic pocket close to Ca-binding loop I makes it an excellent probe for the coupling between folding and calcium binding. In the absence of calcium, the addition of high concentrations of urea promotes a complete red shift in the Trp emission. In the presence of calcium, the complete unfolding of the N-domain of TnC could only be achieved by combining high hydrostatic pressure and urea, which allowed us to determine the thermodynamic parameters. In general, our study goals are to evaluate the relevance of the free-energy change of the N- and C-domains to muscle contraction.

The factors that determine the affinity and selectivity of sites I, II, III, and IV of TnC for Ca²⁺ have been studied extensively (30,31). Using five recombinant chicken TnC fragments, Fredricksen and Swenson (32) documented an inverse correlation between protein stability and affinity for Ca²⁺, and we have confirmed this inverse relationship (33). We observed that a progressive increase in the thermodynamic stability of the N-domain induced by glycerol was accompanied by a decrease in Ca²⁺ affinity. However, this inverse relationship

TABLE 1   Values of volume change (ΔV) obtained at 1°C and 20°C

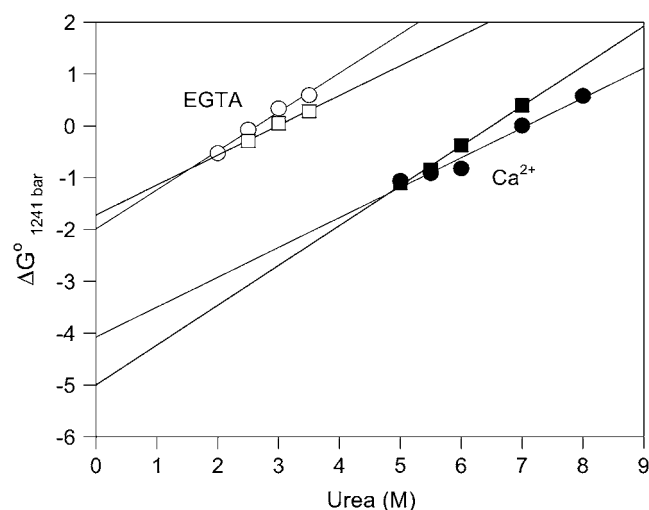|	apo			2.1 mM CaCl₂	
	1°C	20°C		1°C	20°C
Urea (M)	ΔV (ml/mol)	ΔV (ml/mol)	Urea (M)	ΔV (ml/mol)	ΔV (ml/mol)
2.0	–	30.19 ± 0.69	5.0	41.66 ± 0.95	36.74 ± 0.76
2.5	44.82 ± 1.28	38.13 ± 1.73	5.5	48.69 ± 0.74	36.60 ± 1.40
3.0	49.58 ± 1.08	44.24 ± 1.68	6.0	51.04 ± 1.12	46.39 ± 1.12
3.5	49.89 ± 2.03	48.62 ± 2.03	7.0	61.58 ± 1.09	59.59 ± 0.73
			8.0	—	60.65 ± 3.95

Pressure (0–3000 bars) was applied in steps at each urea concentration and the extent of reaction (αp, from the change in center of mass) was plotted according to Eq. 3 to obtain ΔV (ml/mol).

TABLE 2 Experimental volume changes obtained from the pressure dependence of the peak areas for selected peak in the F29W/N-domain ^{1}H-NMR spectra

Peak	ΔV (ml/mol)	Structural description
F22NH	30 ± 2	
K23NH	8 ± 5	
W29NH	15 ± 2	Core
G35NH	42 ± 0.4	Loop I
I37γCH$_3$	30 ± 3	β-sheet, core
S70NH	10.2 ± 0.4	Loop II
G71NH	41.9 ± 0.4	Loop II

TABLE 3 ΔG°_{atm} of folding of N-domain obtained at 1°C and 20°C

	1°C		20°C	
	apo	Ca^{2+}	apo	Ca^{2+}
ΔG°_{atm} (kcal/mol)	-3.07 ± 0.25	-6.67 ± 0.29	-3.40 ± 0.24	-5.82 ± 0.50

Values shown are mean $\pm$ SD ($n = 3$).

has not been found in all situations. Henzl and coworkers (34) observed that introduction of a fifth carboxylate residue at the $+z$ position in the CD coordination sphere or at the $-x$ position in the EF coordination sphere significantly increases the affinity of oncomodulin sites for Ca^{2+}. As shown in Table 4 and observed by Henzl et al. (34), this increase in Ca^{2+} affinity was accompanied by a decrease in thermodynamic stability of S55D, G98D, and 55/98 double mutant apo forms, as determined by differential scanning calorimetry. However, the thermal stability of S55D and its affinity for Ca^{2+} are lower than the corresponding values for the G98D mutant. This behavior would not be predicted from the data of Fredricksen and Swenson (32). In another study, Grabarek et al. (35) observed that acetylation of rabbit skeletal TnC causes a decrease in thermal stability of both domains of TnC in the absence of Ca^{2+}. This decrease in thermal stability was accompanied by a 3.5-fold increase in affinity for Ca^{2+} at the low-

affinity sites, but there was a twofold decrease in affinity at the high-affinity sites.

In this study, by the combined use of high pressure and subdenaturing concentrations of urea, we assessed all the thermodynamic parameters for the folding of the N-domain of TnC in all of its occupancy states. The free-energy change (ΔG°_{atm}) for the folding of the N-domain apo form at 20°C was -3.4 kcal/mol. This value is identical to the one obtained by ^{1}H-NMR, using the F26δ resonance peak change that was induced by high temperatures (27). At 1°C, the ΔG°_{atm} increases, suggesting that lowering the temperature decreases the hydrophobic contacts responsible for part of the stability of the F29W/N-domain. This decrease and consequent lessened stability renders the protein more sensitive to the pressure denaturation in the presence of urea. In contrast, Ca^{2+} binding to the F29W/N-domain increases its stability. It is interesting to note that, at 1°C, Ca^{2+} binding induces greater enhancement of the N-domain stability (Table 3). This behavior is expected, because the Ca^{2+}-binding reaction (N-domain $\Leftrightarrow$ N-domain-Ca$_2$) occurs with a negative ΔG° derived from a large negative enthalpy change and a small change in entropy (36).

The high-pressure NMR experiments were complementary to the fluorescence data because they enabled us to derive values of ΔV for amino acid residues located in distinct regions of the protein structure (Table 2). High-pressure NMR has been successfully used to explore the protein conformation space (20,37,38). It would be expected that the greatest net change in volume would occur for regions that are highly packed in the folded protein, such as the hydrophobic core. Within the core, hydrophobic interactions are maximized, and these interactions are crucial for the proper folding and stabilization of the native state of the protein. Similar to what was

FIGURE 6 Assessing the free-energy change for denaturation of the N-domain in the absence of urea. The ΔV values at 1241 bar obtained from Figs. 4 and 5 are shown in the absence (*open circles* and *open squares*) and in the presence (*solid circles* and *solid squares*) of 2.1 mM CaCl$_2$ at 20°C (*circles*) and at 1°C (*squares*). The ΔG° at 1241 bar in the absence of urea was obtained by extrapolation of these curves to the y axis. The values are the average of three experiments, and error bars were smaller than the symbols.

TABLE 4 Values of pCa$_{50}$ and ΔG°_{atm} for wild-type oncomodulin and its mutants (34)

	pCa$_{50}$ (CD site)	pCa$_{50}$ (EF site)	ΔG°_{atm} (kcal/mol)		$\Delta\Delta G^{\circ}_{atm}$ (kcal/mol)
			apo	Ca^{2+}	
1. WT	6.10	7.35	-3.9	-12.6	-8.7
2. S55D	6.10	7.40	-2.6	-13.1	-10.5
3. G98D	7.17	7.59	-3.2	-15.0	-11.8
4. 55/98	7.35	8.40	-2.4	-15.9	-13.5

$\Delta\Delta G^{\circ}_{atm}$ was calculated by subtracting the value for ΔG°_{atm} of the apo form from that of the Ca^{2+}-bound form.
Temperature: 25°C.

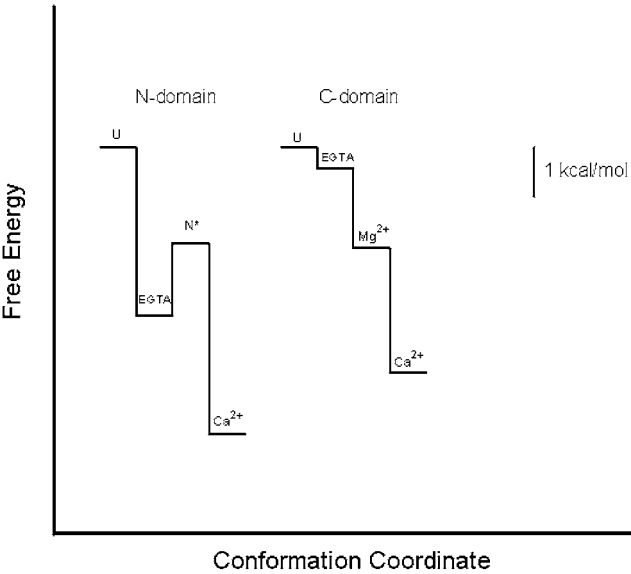FIGURE 7 Thermodynamic diagram for the folding of the N- and C-domains.

TABLE 5 Values of pCa_{50}, ΔG°_{atm}, and $\Delta \Delta G^{\circ}_{atm}$ calculated in this study and in previous articles

| | | ΔG°_{atm} (kcal/mol) | | $\Delta \Delta G^{\circ}_{atm}$ (kcal/mol) |
	pCa_{50}	Ca^{2+}	apo	
1. F105W/ C-domain (20°C)	6.93*	−4.56[‡]	−0.42[‡]	−4.14[‡]
2. F105W (20°C)	6.66*	−4.44[‡]	−0.44[‡]	−4.0[‡]
3. F29W/N-domain (1°C)	6.54[†]	−6.67	−3.07	−3.60
4. F29W/N-domain (20°C)	5.90[†]	−5.82	−3.40	−2.42

*(22).
[†](15).
[‡](14).

whereas addition of Ca^{2+} to the N-domain increases its stability by 2.42 kcal/mol. Thus, the increase in stability gained by the C-domain when Ca^{2+} binds is >50% greater than the gain due to Ca^{2+} binding to the N-domain. The values of ΔG°_{atm} and pCa_{50} for TnC are shown in Table 5. Fig. 8, A and B, shows the plot of $\Delta \Delta G^{\circ}_{atm}$ between the apo and Ca^{2+}-bound states versus pCa_{50} for TnC and oncomodulin, respectively, and demonstrates a good correlation between $\Delta \Delta G^{\circ}_{atm}$ and pCa_{50}. Moreover, the affinity for Ca^{2+} rises when $\Delta \Delta G^{\circ}_{atm}$ increases in absolute terms. Thus, we suggest that part of the affinity for Ca^{2+} comes from the contribution of the free-energy change of folding that takes place when Ca^{2+} binds. The excellent correlation between the processes of protein folding and binding of Ca^{2+} is quite remarkable.

The free-energy diagram shown in Fig. 7 also demonstrates that the apo form of the C-domain is much less structured than the N-domain, which leads to more coupling when Ca^{2+} binds. The linkage between folding and binding has been observed in many proteins, especially when they are unstructured or partially structured (39). In particular, Ca^{2+}-induced changes in protein conformations are the basis for Ca^{2+}-dependent signaling in the cell (40). It is of note that, in the case of TnC, the two domains present different degrees of coupling between
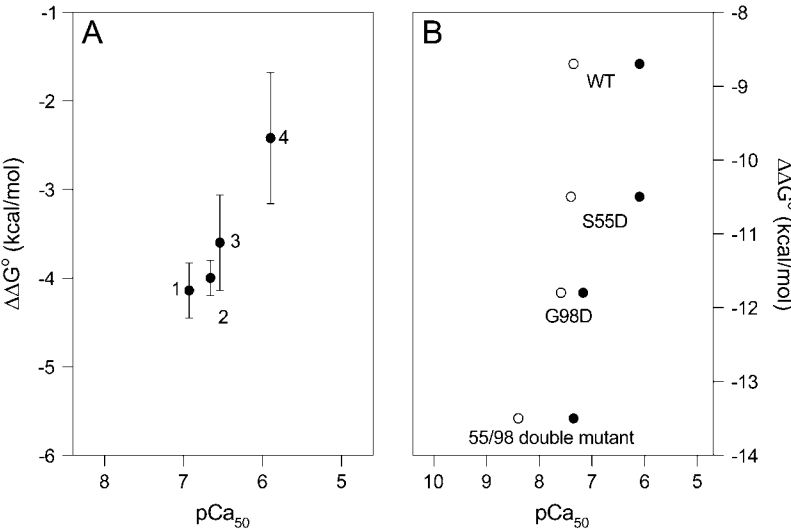
found for the TnC C-domain (14), the largest ΔV values for the TnC N-domain were found for residues located in the hydrophobic core (Table 2), and they are similar to the values derived from the fluorescence experiments. Thus, the thermodynamic values derived from the fluorescence experiment represent the average behavior of the tertiary conformation of the protein, whereas the NMR changes reveal the site-specific changes.

In the free-energy diagram shown in Fig. 7, we compare the free-energy changes for the folding of the N- and C-domains. The free-energy change for folding of the C-domain apo form is very low, confirming its poor stability. Upon Mg^{2+} binding, its stability increases by 1.63 kcal/mol. The addition of Ca^{2+} to the C-domain increases its stability by 4.14 kcal/mol,

FIGURE 8 Plot of $\Delta \Delta G^{\circ}_{atm}$ between apo and Ca^{2+}-bound states versus pCa_{50} for TnC (A) and oncomodulin (B). The values of ΔG°_{atm} and pCa_{50} of TnC are shown in Table 4: 1-F105W/ C-domain (20°C); 2-F105W (20°C); 3-F29W/ N-domain (1°C); 4-F29W/N-domain (20°C). (B) pCa_{50} of EF site (open circles) and pCa_{50} of CD site (solid circles) of oncomodulin versus $\Delta \Delta G^{\circ}_{atm}$. The values of ΔG°_{atm} and pCa_{50} were obtained by Henzl et al. (34).

metal binding and folding. In the case of the N-domain, the higher stability of the apo form might be related to the need to populate a species that exposes a hydrophobic surface when it binds Ca^{2+} (15). This form is represented in the diagram shown in Fig. 7 as an intermediate (N*) that was demonstrated to exist by different experimental techniques (fluorescence intensity changes, Ca^{2+}-binding curves, Trp lifetimes, and bis-ANS binding). This N* intermediate has a nonpolar segment exposed to the aqueous environment and a high affinity for Ca^{2+} (15). Although this conformation can be populated by the combination of pressure and low temperature (2.2 kbar and $-11°C$) and therefore has a high chemical potential, it is still more stable and ordered than the apo TnC/C-domain. The two domains of TnC have evolved to play different roles in the same molecule, each being strictly dependent on coupling, folding, and binding events in the presence of different concentrations of Ca^{2+} or Mg^{2+}, as required for different signaling and background conditions.

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