Role of Hydration in the Closed-to-Open Transition Involved in Ca^{2+} Binding by Troponin C[†]

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ABSTRACT: Troponin C (TnC) is the Ca^{2+}-binding subunit of the troponin complex of vertebrate skeletal muscle. It consists of two structurally homologous domains, N and C, connected by an exposed α-helix. The C-domain has two high-affinity sites for Ca^{2+} that also bind Mg^{2+}, whereas the N-domain has two low-affinity sites for Ca^{2+}. Previous studies using isolated N- and C-domains showed that the C-domain apo form was less stable than the N-domain. Here we analyzed the stability of isolated N-domain (F29W/N-domain) against urea and pressure denaturation in the absence and in the presence of glycerol using fluorescence spectroscopy. Increasing the glycerol concentration promoted an increase in the stability of the protein to urea (0−8 M) in the absence of Ca^{2+}. Furthermore, the ability to expose hydrophobic surfaces normally promoted by Ca^{2+} binding or low temperature under pressure was partially lost in the presence of 20% (v/v) glycerol. Glycerol also led to a decrease in the Ca^{2+} affinity of the N-domain in solution. From the ln K_{obs} versus ln a_{H_2O}, we obtained the number of water molecules (63.5 ± 8.7) involved in the transition $N \Leftrightarrow N{\cdot}Ca_2$ that corresponds to an increase in the exposed surface area of 571.5 ± 78.3 Å^2. In skinned fibers, the affinity for Ca^{2+} was also reduced by glycerol, although the effect was much less pronounced than in solution. Our results demonstrate quantitatively that the stability of this protein and its affinity for Ca^{2+} are critically dependent on protein hydration.

Troponin C (TnC)[1] is the Ca^{2+}-binding subunit of the troponin complex, which contains two other subunits, troponin T and I. Binding of Ca^{2+} to TnC has been shown to induce a conformational change that triggers subsequent events in the initiation of muscle contraction (*1*). The result of this change in conformation is the exposure of a hydrophobic segment and of negatively charged groups surrounding the hydrophobic cleft that serves as a docking site for TnI. This conformational change induced by Ca^{2+} binding has been referred to as the opening of the N-domain (*2*).

The crystal structure of turkey (*3*, *4*), chicken (*5*, *6*), and rabbit TnC (*7*) reveals two similar domains, N and C, separated by a long, exposed α-helix. Recent NMR studies also characterize the globular structure and α-helical content of these two domains, but the central helix was shown to be unstructured in solution (*8*). Each globular domain has two divalent-cation binding sites that consist of a helix−loop−helix motif in which six amino acid residues located in the loop coordinate the bound cation. The sites located in the N-domain (I and II) have low affinity for Ca^{2+} ($K_{a[Ca]} = 3.2 \times 10^5$ M^{-1}), whereas the sites in the C-domain (III and IV) have high affinity for Ca^{2+} ($K_{a[Ca]} = 2 \times 10^7$ M^{-1}) and also bind Mg^{2+} ($K_{a[Mg]} = 5 \times 10^3$ M^{-1}).

Several studies have been performed to explain the differences in Ca^{2+} affinity of the N- and C-domains. The importance of each amino acid in the Ca^{2+}-binding loop has been evaluated by single amino acid substitutions in the intact protein, as well as by the use of synthetic peptides that correspond to the Ca^{2+}-binding sites (helix−loop−helix motif). As expected, drastic changes in affinity are caused by changing the amino acids involved in Ca^{2+} coordination (*9*−*12*). Additionally, the affinity for Ca^{2+} seems to be affected by more than just the amino acid sequence of the Ca^{2+}-binding loop. Reid et al. (*13*) and Monera et al. (*14*) used synthetic peptides to show that long-range interactions can also affect Ca^{2+} affinity. Grabarek et al. (*15*) and Fujimori et al. (*16*) observed a decrease in the Ca^{2+} affinity when single-site replacements were used to form covalent or ionic links between adjacent helices in positions distant from the Ca^{2+} coordinating residues; the effect was to render the N-domain less able to open upon Ca^{2+} binding. In the C-domain of chicken TnC, replacing Ile with Thr at position 130, more than 20 Å from the divalent cation in sites III

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[1] Abbreviations: Bis-ANS: 4,4′-bis(1-anilino-8-naphthalenesulfonic acid); DTT: dithiothreitol; EGTA: ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; TnC: troponin C; TnI: troponin I.

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and IV, increases Ca^{2+} affinity by a factor of 2.5 (*17*). Removal of the N-helix (11 amino acids that precede helix A at the N-terminus) significantly reduces Ca^{2+} affinity of the N-domain (*18*). Foguel et al. (*19*) observed that sites I and II of the N-domain have a higher affinity for Ca^{2+} at 1 °C than at 20 °C. They also showed that a decrease in temperature under pressure promotes spectroscopic alterations in the N-domain that mimic those caused by Ca^{2+} binding. These results suggest that low temperatures under pressure induce the opening of the N-domain by destabilizing intradomain hydrophobic interactions.

The thermodynamic stability of intact TnC (*20*) as well as that of its isolated domains (*21*, *22*) has been investigated by several groups. In the absence of added cations, C-domain exhibits a decrease in stability compared to the N-domain when either heat or chemical agents are used as denaturants. In these studies, it was also pointed out that there is an inverse correlation between stability and Ca^{2+} affinity. Studying the properties of TnC fully acetylated at its lysine residues, Grabarek et al. (*21*) observed a decrease in the thermal stability of N-domain and a 3.5-fold increase in the affinity for Ca^{2+}. In a recent report, Fredricksen and Swenson (*22*) used five recombinant chicken TnC fragments and detected an inverse correlation between Ca^{2+} affinity and protein stability when the latter was measured in the absence of cations.

On the other hand, Ca^{2+} binding to the N- and C-domains induces an enormous increase in stability, as exemplified by a shift in the T_m for unfolding the N-domain by approximately 58 °C at neutral pH (*20*). This great gain in stability makes it difficult if not impossible to achieve a direct measurement of the free energy change of folding for the N- and C-domains in the ion-bound forms.

In this study, using recombinant TnC N-domain (F29W/N-domain, residues 1−90) and intact TnC, we have investigated the effects of decreasing water content of the solution on the stability, Ca^{2+} affinity and physiological function of the N-domain. Glycerol (5−20%) was used as a cosolvent to reduce water activity. Previous studies have shown that glycerol increases the stability of several proteins (*23*−*26*). Here we show that glycerol counteracts urea-induced unfolding of the N-domain in a dose-dependent fashion. Complete unfolding of the N-domain is achieved by combining high hydrostatic pressure and urea, a maneuver that allows us to calculate the free-energy change for folding of the N-domain in the presence and absence of glycerol. This increase in the stability of the N-domain promoted by glycerol is accompanied by a concomitant decrease in Ca^{2+} affinity. The opening of the N-domain induced by Ca^{2+} binding or by lowering the temperature under pressure (*19*) is also inhibited by glycerol, suggesting that water is necessary to wet the exposed surface of the N-domain upon opening.

EXPERIMENTAL PROCEDURES

Sample Preparation. The N-domain mutant (F29W/N-domain, residues 1−90) was prepared as described in Li et al. (*27*). All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system. The experiments under pressure were performed in the standard buffer (100 mM Tris-HCl, 100 mM KCl, 1.0 mM DTT, and 1.5 mM EGTA, pH 7.0). Glycerol was added to this buffer at the desired concentrations, replacing water. The concentration of N-domain was 5 μM, unless otherwise stated in the legends of the figures.

Calcium Titration Curves. Calcium titration curves were obtained from the Ca^{2+}-induced increase in fluorescence intensity at 336 nm (*27*), using an apparent K_a of 1.92×10^6 M^{-1} for the calcium−EGTA complex. The solution contained 50 mM MOPS, 100 mM KCl, 1 mM DTT, and 1 mM EGTA, at pH 7.0. Calcium titration did not alter the pH. Glycerol was added to this solution when stated.

Fluorescence Measurements under Pressure. Paladini and Weber (*28*) have described the high-pressure cell. The bomb was kept at different temperatures with the aid of a circulating water bath, while a dry nitrogen gas flush prevented water condensation on the optical surfaces. The tryptophan and bis-ANS fluorescence spectra were recorded on an ISSK2 spectrofluorometer (ISS Inc., Champaign, IL). Tryptophan emission was measured by exciting the samples at 280 nm and collecting the emission from 300 through 400 nm, after equilibration for 5 min. All spectroscopic changes reported here were completely reversible upon pressure removal, suggesting that the unfolding reaction is also reversible. Bis-ANS binding was evaluated from the area of the emission spectra where excitation was set at 360 nm and emission scanned from 400 to 600 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 $\nu_{p,u}$, in cm^{-1}:

$$\nu_{p,u} = \Sigma \nu_i F_i / \Sigma F_i \quad (1)$$

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

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

$$K_f(p) = K_{atm} \exp(p\Delta V/RT) \quad (2)$$

where $K_f(p)$ and K_{atm} are the equilibrium constants of folding at pressure p and atmospheric pressure, respectively. If we introduce the extent of reaction at pressure p, α_p, we deduce the following thermodynamic relation (*29*, *30*):

$$\ln(\alpha_p/(1 - \alpha_p)) = p(\Delta V/RT) + \ln K_{atm} \quad (3)$$

The complete unfolding of the N-domain was attained only when high pressure was combined with subdenaturing concentrations of urea. However, the volume change (ΔV) increased linearly with urea addition, leveling off at the highest concentration of urea used (Tables 1 and 2). This kind of behavior makes the use of the direct relation between the standard free energy of folding ($\Delta G°$) 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 4). The extrapolation of this plot to 0 M urea gives $\Delta G^{o0M}{}_{1241}$. This value was introduced into eq 5 to determine the free energy change at atmospheric pressure (ΔG^{o}_{atm}).

$$\Delta G^{o[U]}_{1241} = m[U] + \Delta G^{o0M}_{1241} \quad (4)$$

Table 1: Volume Change (ΔV) of N-Domain Apo Form Obtained in the Absence and Presence of 5, 10, and 20% Glycerol at 20 °C

	ΔV (mL/mol)[b]			
urea (M)	without glycerol[a]	5% glycerol	10% glycerol	20% glycerol
2.0	30.19 ± 0.69			
2.5	38.13 ± 1.73	32.59 ± 1.64	33.53 ± 1.25	
3.0	44.24 ± 1.68	36.06 ± 1.59	43.93 ± 1.09	26.84 ± 0.80
3.5	**48.62 ± 2.03**	50.61 ± 1.36		32.98 ± 1.41
4.0		**44.10 ± 3.77**	**53.98 ± 1.08**	**43.36 ± 1.64**

[a] Suarez et al., unpublished. [b] Pressure (0−3000 bar) was applied in steps at each urea concentration and the extent of reaction (α_p, from the change in center of mass) was plotted according to eq 3 (Experimental Procedures) to obtain ΔV.

Table 2: Volume Change (ΔV) of N-Domain Ca^{2+}-Bound Form Obtained in the Absence and Presence of 5, 10, and 20% Glycerol at 20 °C

	ΔV (mL/mol)[b]			
urea (M)	without glycerol[a]	5% glycerol	10% glycerol	20% glycerol
5.0	36.74 ± 0.76		31.37 ± 2.19	
6.0	49.39 ± 1.12	51.95 ± 1.14	34.76 ± 0.44	21.70 ± 0.90
6.5			**53.18 ± 0.71**	
6.7				26.18 ± 0.67
7.0	59.59 ± 0.73	56.59 ± 1.34		
7.5				**45.29 ± 2.76**
7.9		**58.03 ± 2.15**		
8.0	**60.65 ± 3.95**			

[a] Suarez et al., unpublished. [b] Experiments were performed at 0−3000 bar as described for Table 1, but in the presence of 2.1 mM $CaCl_2$.

$$\Delta G^{o0M}_{1241} = \Delta G^{o}_{atm} + p\Delta V \quad (5)$$

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 the difference in solvent-accessible surface area as the protein unfolds (*31*). The volume change used in eq 5 was the highest value observed (marked in bold in Tables 1 and 2). We chose 1241 bar because this pressure is close to the p_{50} values of the majority of the curves.

The thermodynamic approach we have used to calculate ΔV and $\Delta G°$ assumes that Ca^{2+} remains bound to the N-domain under high pressure. We have no direct experimental evidence for this assumption, but there are reports in the literature stating that calcium remains bound to the N-domain even in the presence of high concentrations of urea (*32*, *33*). Thus, we assume that a first-order equilibrium (N ↔ U) is established under pressure without taking into consideration the dissociation of Ca^{2+}.

Osmotic Stress Approach. The data from the Ca^{2+}-titration curves with and without glycerol were interpreted in terms of the osmotic stress approach (*34*, *35*). According to this approach, the binding of Ca^{2+} to the N-domain (N) in aqueous solution containing glycerol (G) would be represented by

$$2\ Ca^{2+} + N \leftrightarrow [Ca_2{:}N] + \upsilon_{H_2O} + \upsilon_G \quad (6)$$

in which υ_{H_2O} and υ_G represent, respectively, changes in the stoichiometric amounts of water and glycerol binding to protein upon calcium−N-domain complex formation. If experiments are performed under isothermal and isobaric conditions, and in the presence of a neutral solute (e.g., glycerol), changes in the equilibrium binding constant (K_{obs}) for [Ca_2:N] complex formation in eq 7 can be expressed as

$$d \ln K_{obs} = (\partial \ln K_{obs}/\partial \ln a_{H_2O}) d \ln a_{H_2O} + (\partial \ln K_{obs}/\partial \ln a_G) d \ln a_G \quad (7)$$

According to Wyman's linkage relation (*36*),

$$(\partial \ln K_{obs}/\partial \ln a_i) = \upsilon_i \quad (8)$$

in which υ_i is the change in the number of component i bound to the protein upon formation of the complex. Combining eqs 7 and 8 we obtain

$$(d \ln K_{obs}/d \ln a_{H_2O}) = \upsilon_{H_2O} + \upsilon_G\ (d \ln a_G/d \ln a_{H_2O}) \quad (9)$$

Glycerol is preferentially excluded from the immediate vicinity of the protein (*37*). In this sense, as changes in the chemical potential of water and glycerol are directly coupled (*38*), we consider glycerol as an agent that adjusts the chemical potential of water, and so, if there is a large change in the number of solvent molecules on the protein surface upon complexation υ_{H_2O} is significantly larger than υ_G and thus, υ_{H_2O} contributes more importantly to $\partial \ln K_{obs}$ than does υ_G.

Water activity ($\ln a_{H_2O}$) is inversely proportional to solute osmolal concentrations ($\ln a_{H_2O} = -$[solute]/55.6). Osmolal concentration of glycerol was calculated from tabulated data (*39*).

Glycerol (5−20%) was chosen as cosolvent since it reduces water activity, without affecting drastically either water viscosity or the macroscopic dielectric constant of buffer solutions (*23*, *24*, *40*).

Circular Dichroism Measurements. Circular dichroism (CD) measurements were performed in a Jasco-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) using a 1.0-mm path-length 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, pH 7.0, in the absence or in the presence of 2.1 mM $CaCl_2$ and glycerol (concentrations are in % v/v, unless otherwise stated). Data were averaged for two scans at a speed of 50 nm/min, collected in 0.2 nm steps. The baselines (buffer alone) were subtracted.

Skinned-Fiber Assays. Rabbit psoas fibers were skinned at 0 °C and stored at −20 °C in skinning solution containing 50% glycerol (*41*). Protease inhibitors were added to skinning and storage solutions. Single fiber segments were dissected in relaxing solution, rinsed to remove glycerol, and mounted on an Akers transducer as described previously (*42*, *43*). The experiments were performed in a stirred solution containing 152 mM potassium propionate, 20 mM imidazole, 4 mM $MgATP^{2-}$, 5 mM K_2EGTA-CaEGTA, and 2 mM free Mg^{2+}, pH 7.0, in the absence or in the presence of 20% glycerol, at 15 °C. CaEGTA replaced part of the K_2EGTA to give the free Ca^{2+} concentrations indicated in Figure 9, while keeping the total EGTA concentration constant. Data points on the tension−pCa curve for each fiber were fit using the equation

$$P/P_0 = 100[Ca]^n/((K_{0.5})^n + [Ca]^n) \quad (10)$$

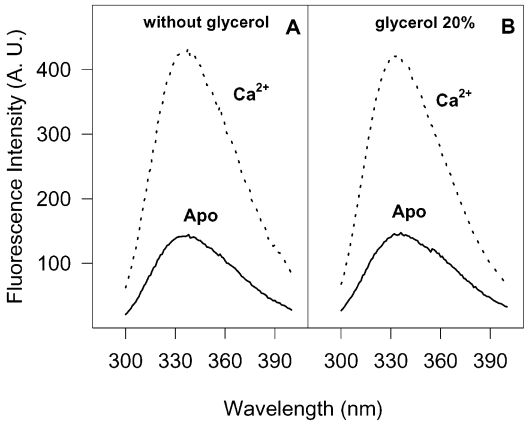

FIGURE 1: Glycerol does not affect the tryptophan emission spectra of F29W/N-domain. F29W/N-domain (5 μM) in the absence (−) and in the presence of 2.1 mM $CaCl_2$ (···). In (A), without glycerol and in (B), in the presence of 20% glycerol. The samples were excited at 280 nm and the emission was measured from 300 to 400 nm. The solvent conditions were 100 mM Tris-HCl, pH 7.0, 100 mM KCl, 1 mM DTT, and 1.5 mM EGTA, at 20 °C.

and the values reported for each $K_{0.5}$ (midpoint of the curve) and n (Hill n) are the average ± standard error for five fibers.

RESULTS

Effects of Glycerol on the Spectroscopic Properties of F29W/N-Domain. The intrinsic fluorescence of Trp 29 was used to measure the effect of Ca^{2+} binding to F29W/N-domain, in the absence and in the presence of increasing concentrations of glycerol. Upon Ca^{2+} binding, there is a 3-fold increase in Trp 29 emission at 336 nm (Figure 1 and *27*). Initially, we investigated whether adding glycerol (5, 10, and 20%) would interfere with the spectroscopic properties of Trp 29 upon Ca^{2+} binding. Figure 1 shows that the addition of 20% glycerol did not change the effect of Ca^{2+} on the Trp29 emission spectrum, and neither did 5 or 10% glycerol (data not shown). In all conditions, the fluorescence intensity at 336 nm increased around 3-fold on going from apo to Ca^{2+}-bound forms (Figure 1). The spectrum of the apo form was also unchanged by glycerol.

It is well established that binding of Ca^{2+} to TnC induces an increase in negative ellipticity that can be attributed partially to an increase in its α-helical content (*27*). Figure 2 shows that 20% glycerol does not alter the gain in ellipticity promoted by maximal Ca^{2+} binding. Taken together, these results suggest that the native structure of the N-domain is not affected by the presence of glycerol and neither are the spectroscopic changes that take place upon maximal Ca^{2+} binding.

The Thermodynamic Stability of the N-Domain in the Absence and in the Presence of Glycerol. Fredricksen and Swenson (*22*) described an inverse correlation between stability and Ca^{2+} affinity using five recombinant chicken TnC fragments. Here, we also investigated if changes in the thermodynamic stability of the N-domain induced by glycerol would be accompanied by an increase or decrease in Ca^{2+} affinity. With this aim, we monitored denaturation of F29W/N-domain by measuring changes in the center of spectral mass of Trp 29 induced by increasing concentrations of urea (0 to 8 M) (Figure 3). A decrease in the center of spectral mass correlates with Trp exposure to the aqueous environment, and a value close to 28 400 cm^{-1} represents a fully

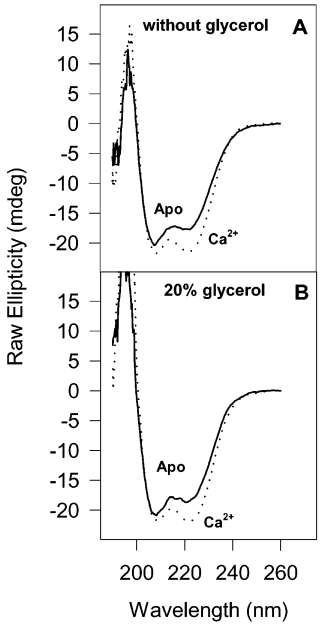

FIGURE 2: Glycerol does not affect the negative ellipticity of F29W/N-domain. The far-UV CD spectra of F29W/N-domain (12 μM) in the absence (−) and in the presence of 2.1 mM $CaCl_2$ (···). In (A), without glycerol and in (B), in the presence of 20% glycerol. The solvent conditions were 10 mM Tris-HCl, pH 7.0, 100 mM KCl, 1 mM DTT, and 1.5 mM EGTA.

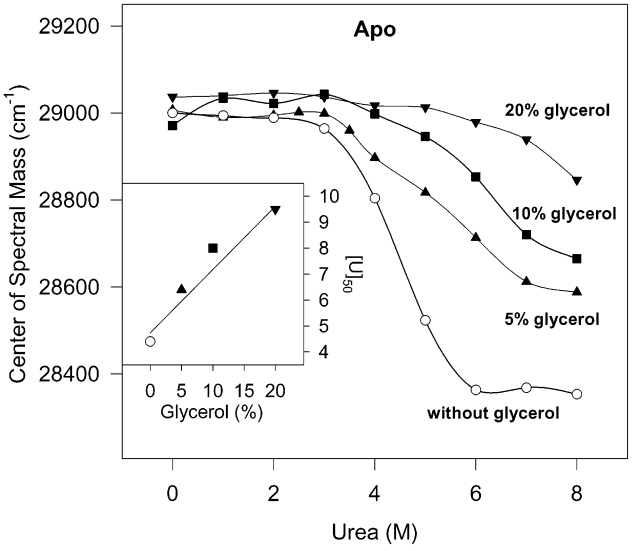

FIGURE 3: Glycerol increases the stability of F29W/N-domain apo form toward urea. Effect of urea on the center of spectral mass of F29W/N-domain in the absence (○) and in the presence of 5 (▲), 10 (■), and 20% glycerol (▼). Inset: Half-maximal point of each curve (molar concentration of urea, $[U]_{50}$) against glycerol concentration. All other conditions were as described in Figure 1.

exposed Trp residue. We observed that while the apo N-domain in the absence of glycerol was completely denatured by urea up to 6 M, addition of glycerol displaced the unfolding curve to higher urea concentrations, suggesting stabilization due to changes in water activity modulated by glycerol. In the presence of 5, 10, or 20% (v/v) glycerol, even 8 M urea was unable to promote complete denaturation of F29W/N-domain. The $[U]_{50}$ (concentration of urea necessary to promote half-maximal denaturation) was progressively displaced to higher urea values (Figure 3, inset). In the presence of 20% glycerol, by extrapolation 50% unfold-

Table 3: Free Energy Change of Folding (ΔG^{o}_{atm}) Obtained in the Absence and Presence of 5, 10, and 20% Glycerol at 20 °C[a]

	ΔG^{o}_{atm} (kcal/mol)			
	apo form	0.6 mM Ca^{2+}	$\Delta\Delta G^{o}$ (kcal/mol)	pCa_{50}
without glycerol[b]	-3.40 ± 0.24	-5.82 ± 0.50	-2.42 ± 0.74	5.70 ± 0.40
5% glycerol	-4.12 ± 0.22	-6.50 ± 0.41	-2.38 ± 0.63	5.10 ± 0.17
10% glycerol	-4.23 ± 0.2	-6.83 ± 0.06	-2.60 ± 0.26	4.60 ± 0.31
20% glycerol	-5.58 ± 0.05	-7.80 ± 0.10	-2.22 ± 0.15	3.79 ± 0.08

[a] The $\Delta\Delta G^{o}$ (kcal/mol) between the Ca^{2+} and apo forms and pCa_{50} are also shown. [b] Suarez et al., unpublished results

ing of the N-domain would require 9.5 M urea. This pronounced stabilization in glycerol made it impossible to achieve the unfolded state using only urea, and we were unable to calculate the free energy change of folding caused by urea when glycerol was present. Thus, as described below, we employed a combination of perturbing agents (pressure + urea) to shift the equilibrium of the apo N-domain to the unfolded state (first column of data in Table 3).

A stabilizing effect of Ca^{2+} on the N-domain structure also occurs, likewise making it impossible to assess the free energy change of folding for the N-domain in the Ca^{2+}-bound form by using only chemical agents (*20*). To overcome this large gain in stability due to Ca^{2+} binding, the additive effects of high hydrostatic pressure and a subdenaturing concentration of urea were employed successfully to unfold the N-domain of TnC (Suarez et al., unpublished results). This combination allowed us to calculate the thermodynamic parameters of folding of the N-domain in the presence of Ca^{2+}. These results, to be described in detail elsewhere, are summarized in Table 3 for comparison with the data obtained in the presence of glycerol.

Taking advantage of the additive effects of high hydrostatic pressure and urea, similar experiments were performed in the presence of glycerol. Figure 4 shows the unfolding of N-domain induced by pressure in the presence of 5% glycerol in the Ca^{2+}-bound (panel A) or apo state (panel B) as revealed by the decrease in the center of spectral mass. Similar experiments were performed in the presence of 10 and 20% glycerol (not shown). Applying pressure on the apo N-domain in the absence of added urea produced no changes in the center of spectral mass of Trp29 (not shown). Increasing urea concentrations in the solution facilitated the unfolding of the N-domain. In the presence of Ca^{2+} (panel A), the concentrations of urea (6−8 M) needed to promote the complete unfolding of the N-domain were higher than the concentrations needed to denature the apo state (2.5−4 M; panel B). From the pressure denaturation curves in the presence of increasing concentrations of urea presented in Figure 4, the volume changes (ΔV) of folding of the N-domain in the absence and presence of glycerol and Ca^{2+} were calculated using eq 3 from Experimental Procedures (Tables 1 and 2, respectively).

In order to calculate the ΔG^{o}_{atm} for the folding of the N-domain in different conditions, we first obtained the free energy change at 1241 bar in the presence of different concentrations of urea. This approach was employed in previous studies (*44*, *45*) and is useful when the ΔV values are not constant; as shown in Tables 1 and 2, ΔV increases with urea concentration. In the inset to Figure 4, the extrapolation of these $\Delta G^{o[U]}_{1241}$ values to the ordinate furnishes $\Delta G°$ at 1241 bar in the absence of urea (ΔG^{o0M}_{1241}).

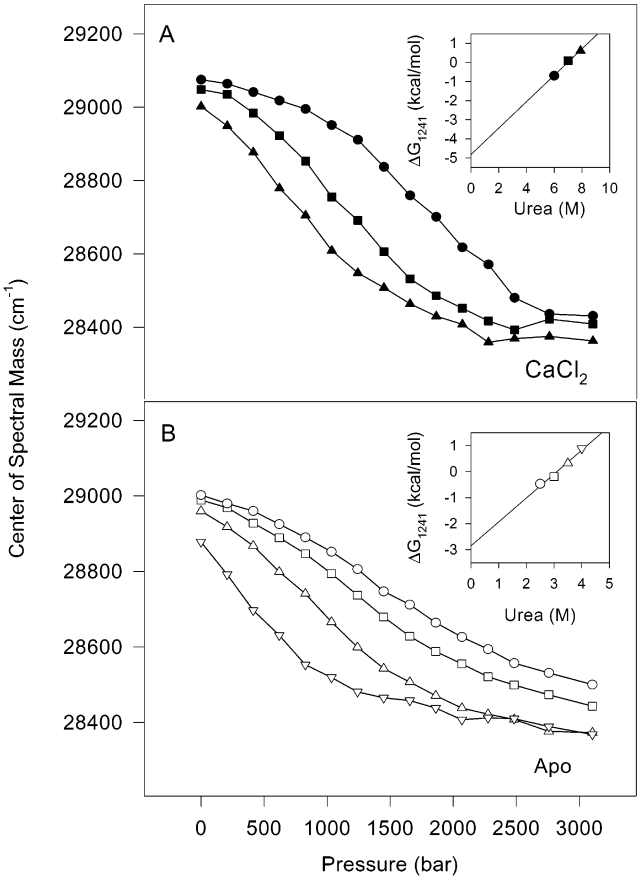

FIGURE 4: High hydrostatic pressure and urea promote denaturation of F29W/N-domain (decrease in center of spectral mass), in the presence of 5% glycerol. In (A), the pressure curves were carried out in the presence of 2.1 mM $CaCl_2$ plus urea: 6.0 M (●), 7.0 M (■), and 7.9 M (▲). In (B), Ca^{2+} was absent and urea was 2.5 M (○), 3.0 M (□), 3.5 M (Δ), and 4.0 M (▽). Inset: $\Delta G^{[U]}_{1241}$ versus urea concentration. The $\Delta G°$ at 1241 bar in the absence of urea was obtained by extrapolation to the ordinate (see text in Results). The protein concentration was 5 μM. All other conditions were as described for Figure 1. The center of spectral mass is completely recovered upon pressure release (not shown).

The free energy change of folding at atmospheric pressure (ΔG^{o}_{atm}) was obtained from eq 5, using the volume changes (ΔV values marked in bold in Tables 1 and 2) from the pressure curves and the ΔG at 1241 bar in the absence of urea. Table 3 shows that ΔG^{o}_{atm} for the N-domain becomes more negative with increasing concentrations of glycerol. Note that the binding of Ca^{2+} to the N-domain increases its thermodynamic stability by −2.42 kcal/mol, a value very similar to that observed when 20% glycerol is added to the N-domain in the apo state (−2.18 kcal/mol, from lines 1 and 4 in column 1 of Table 3). Thus, we can conclude that 20% glycerol stabilizes the N-domain as much as does the binding of Ca^{2+} to sites I and II, although certainly a different

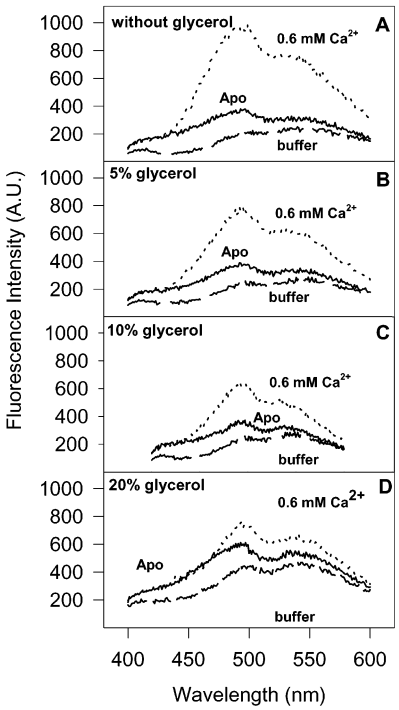

FIGURE 5: Glycerol decreases bis-ANS binding in the presence of Ca^{2+}. Fluorescence emission spectra of bis-ANS in the absence of protein (- -), in the presence of 5 μM F29W/N-domain (−) and in the presence F29W/N-domain plus 2.1 mM $CaCl_2$ (···). In (A), without glycerol. In (B), (C) and (D), in the presence of 5, 10, and 20% glycerol, respectively. The samples were excited at 360 nm and the emission measured from 400 to 600 nm. The protein and bis-ANS concentrations were both 5 μM. All other conditions were as described for Figure 1.

mechanism is involved. Table 3 also shows that a further increase in stability of ca. −2 kcal/mol occurs when Ca^{2+} is added, even in the presence of glycerol.

Effects of Glycerol on the Opening of the N-Domain. Binding of Ca^{2+} to sites I and II induces opening of the N-domain, exposing a hydrophobic region that normally lies buried among the five α-helices of this domain, and changes the interaction between TnC and TnI (*43*, *44*). This conformational change was predicted in the model of Herzberg et al. (*4*) and has been confirmed by NMR spectroscopy and fluorescence studies (*8*, *19*, *48*). The exposure of hydrophobic surface promoted by Ca^{2+} binding can also be detected using bis-ANS. This hydrophobic probe has a marginal fluorescence when free in solution that increases upon binding to hydrophobic surfaces of proteins (*49*, *50*). Figure 5 shows that apo N-domain in the absence of glycerol (panel A) binds very little bis-ANS (the fluorescence is low, middle curve) but that binding increases when Ca^{2+} is added (upper curve). However, in the presence of increasing concentrations of glycerol (panels B, C, and D), the extent of bis-ANS binding upon Ca^{2+} addition progressively decreases. In the presence of 20% glycerol (panel D), the increase in bis-ANS fluorescence area induced by Ca^{2+} binding is only 53% of that observed in the absence of glycerol. This result suggests that glycerol is impeding the opening of the N-domain induced by Ca^{2+} binding.

Previously, we described a conformational state of N-domain induced by low temperature (−11 °C) under pressure, and showed that this state mimics the Ca^{2+}-bound form (the opened N-domain) (*19*). Under pressure at −11 °C, the

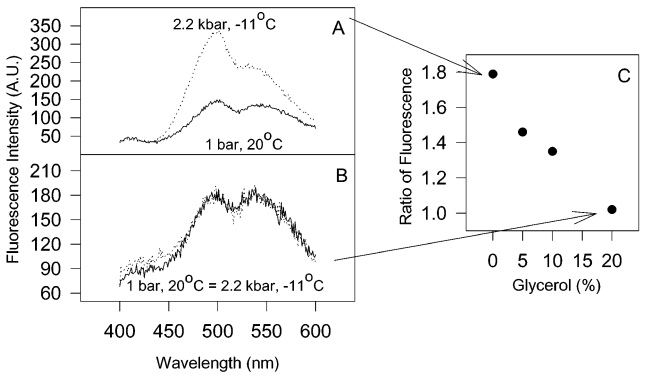

FIGURE 6: Glycerol decreases bis-ANS binding at −11 °C, under pressure. Fluorescence emission spectra of bis-ANS in the presence of F29W/N-domain (5 μM) at atmospheric pressure and 20 °C (−) and at 2.2 kbar and −11 °C (···), in the absence (A) or in the presence of 20% glycerol (B). In (C), ratio of bis-ANS spectral area (2.2 kbar, 11 °C /1 kbar, 20 °C) at each glycerol concentration.

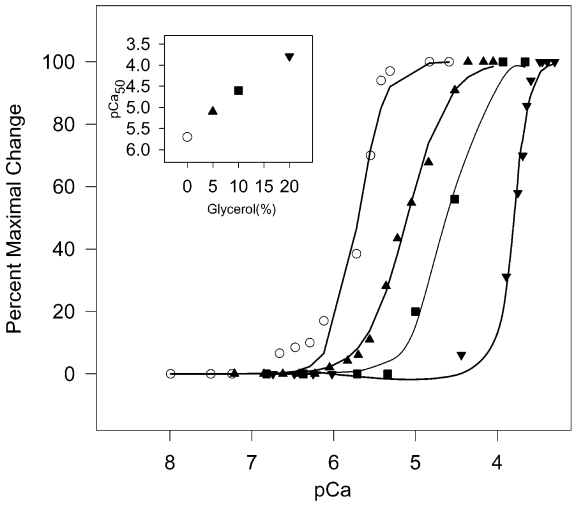

FIGURE 7: Glycerol decreases the Ca^{2+} affinity of F29W/N-domain of troponin C. Ca^{2+} titration curves in the absence (○) or in the presence of 5 (▲), 10 (■), and 20% glycerol (▼). Inset: pCa_{50} of F29W/N-domain versus glycerol concentration. The protein concentration was 5 μM. The samples were excited at 280 nm and the emission was monitored at 336 nm. The buffer contained 50 mM MOPS, 100 mM KCl, 1 mM DTT, and 1 mM EGTA, pH 7.0. The data were fitted with the Hill equation $\theta_n = ([Ca^{2+}]^n/K_{obs}^n)/(1 + ([Ca^{2+}]^n/K_{obs}^n))$; were n is the cooperativity index, $[Ca^{2+}]$ is the free calcium concentration and K_{obs} is the dissociation constant (*51*).

binding of bis-ANS to the apo N-domain is very pronounced due to the extensive exposure of hydrophobic surface to the aqueous environment (Figure 6A) under these conditions. In a similar experiment in the presence of 20% glycerol (Figure 6B), bis-ANS binding to the protein is completely abolished. An intermediate reduction in binding occurs in the presence of 5 and 10% glycerol (Figure 6C). These results suggest that the conformational change induced by lowering the temperature under pressure, although it resembles the opening of the N-domain by Ca^{2+}, is impaired by glycerol. The exposed surface of the N-domain needs water for its hydration and the reduction of water activity by the presence of glycerol may make this conformational change difficult.

Glycerol Decreases the Ca^{2+} Affinity in Solution and in Skinned Fibers. As shown in Figure 7, addition of 5, 10, or 20% (v/v) glycerol progressively decreases the affinity of sites I and II for Ca^{2+}. The pCa_{50} (−log Ca^{2+} at the midpoint

Table 4: Hill Coefficient and Ca^{2+} Affinity of N-Domain in the Absence and Presence of Glycerol[a]

	without glycerol	5% glycerol	10% glycerol	20% glycerol
Hill coefficient	2.73 ± 0.48	1.73 ± 0.09	1.89 ± 0.21	4.51 ± 0.38
$K_{d[Ca]}$ (10^{-6} M)	2.00 ± 0.142	7.91± 0.27	24.9 ± 1.68	160 ± 0.034

[a] Data from experiments such as those of Figure 7.

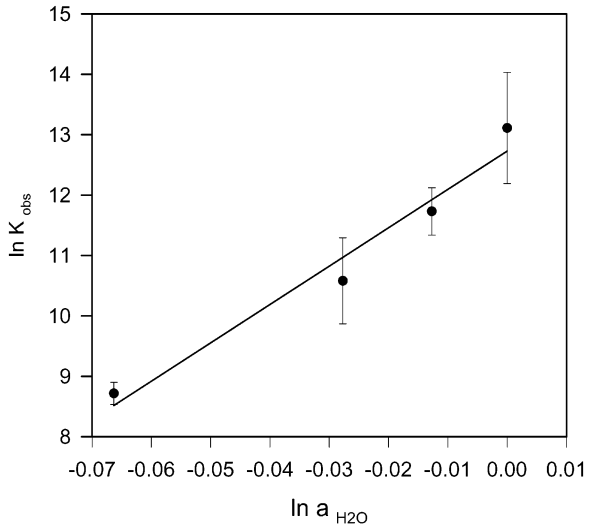

FIGURE 8: Hydration changes in TnC upon Ca^{2+} binding. Linkage plot of ln K_{obs} versus ln a_{H_2O}. The binding constants were determined as in Figure 7.

of the curve) changes from 5.70 to 3.79 as the glycerol concentration increases from 0 to 20% (v/v) (Figure 7, inset). We also observed an increase in the Hill coefficient with 20% glycerol (Table 4). However, the light scattering of N-domain did not change (not shown), suggesting that the protein remains a monomer, even at the highest concentration of glycerol. The decrease in Ca^{2+} affinity with a concomitant increase in stability in the presence of glycerol is in accordance with Fredricksen and Swenson (*22*).

In Figure 8 is plotted the ln K_{obs} versus ln a_{H_2O}, which allows us to calculate the accessible surface area induced by Ca^{2+} binding. From the slope of the curve, we can calculate that 63.5 ± 8.7 water molecules are involved in the conformational change that takes place upon Ca^{2+} binding. Considering that the area of one molecule of water is 9 Å^2 (*34*), we infer an increase in the exposed surface area upon calcium binding of 571.5 ± 78.3 Å^2.

Finally, we analyzed the effects of glycerol on skinned fibers. The affinity for Ca^{2+} in this case was also reduced by the addition of 20% glycerol, although the effect was much less pronounced than in solution (Figure 9 and Table 5). This difference from the data obtained with the isolated protein may reflect the presence of other components of the thin filament in skinned fibers.

DISCUSSION

TnC is one of most studied proteins of the EF-hand family. The factors that determine the affinity and selectivity of sites I, II, III, and IV for Ca^{2+} have been investigated extensively. The site affinity seems to be affected by more than just the amino acid composition of the Ca^{2+} binding loop (*13*, *15*, *21*). In 1996, Fredricksen and Swenson (*22*) detected an

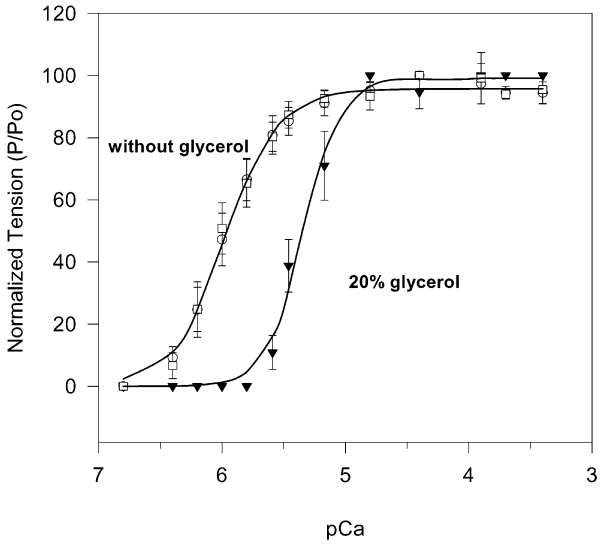

FIGURE 9: Glycerol decreases the Ca^{2+} affinity of skinned fibers. Isometric tensions were measured at 15 °C in 152 mM K-propionate, 20 mM imidazole, 4 mM $MgATP^{2-}$, 2 mM Mg^{2+}, 5 mM EGTA+CaEGTA, pH 7.0, with 0 (○) or 20% (▼) glycerol replacing part of the water. Curves were fit by eq 10 (Experimental Procedures), normalized to the maximum value (P_0) for each curve. Error bars show standard errors for five fibers. After exposure of the sample to glycerol, measurements were repeated in the absence of glycerol (also shown as (□)). Maximal tensions were 42 ± 6.3% of the original in the presence of glycerol and in the second control curve were 95 ± 4.3% of the first.

Table 5: Glycerol Decreases pCa_{50} (−log $[Ca]_{0.5}$) in Skinned Fibers[a]

additions	pCa_{50}	n_H	P/P_0
without glycerol	6.0 ± 0.11	2.36 ± 0.25	100 ± 5.2%
20% glycerol	5.3 ± 1.02	3.79 ± 0.71	43.3 ± 1.2%

[a] Data from experiments of Figure 9.

inverse correlation between protein stability and affinity for Ca^{2+} using five recombinant chicken TnC fragments. This inverse relationship is also observed when the two domains of TnC are compared. The C-domain has two high-affinity sites and is very unstable at 25 °C in the apo state, whereas the N-domain presents two low-affinity sites for Ca^{2+} and shows high structural stability (*20*).

Here we have shown that increasing concentrations of glycerol progressively decrease the affinity of both isolated N-domain (Figure 7) and skinned fibers (Figure 9) for Ca^{2+}, while there is a proportional increase in the stability (Figure 3). These data confirm the observation of Fredricksen and Swenson (*22*). We also observed that urea could only induce complete denaturation in the presence of glycerol when high hydrostatic pressure was applied (Figure 4). Under these conditions, the denatured conformation of the N-domain as measured by Trp emission in the presence of 5, 10, and 20% glycerol is similar to the one obtained in the absence of glycerol.

An additional effect of glycerol in solution was observed in Figures 5 and 6. This osmolyte seems to induce a "locked" conformation of the N-domain that prevents it from opening upon Ca^{2+} binding or when the temperature is lowered under pressure. It may be that the inhibition of the opening of the N-domain contributes to the decrease in Ca^{2+} affinity observed in the presence of glycerol. TnC mutants that favor

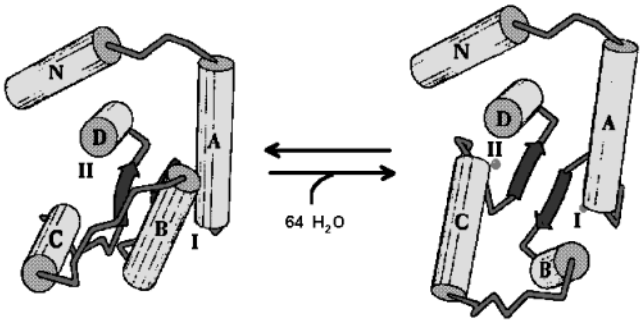

FIGURE 10: Structural model of TnC/N-domain in the absence (left) or in the presence of Ca^{2+} (right) showing the two Ca^{2+}-binding sites (I and II). Structure based on published coordinates for residues 1−90 (*3*). The Ca^{2+} is shown as spheres. The number of water molecules involved in the transition $N \leftrightarrow N{\cdot}Ca_2$ was calculated from the data presented in Figure 8.

the opening of a hydrophobic cleft in the N-domain had increased affinity for Ca^{2+} (*51*, *52*). Conversely, mutants in which the N-domain opening was impeded or blocked had reduced affinity for Ca^{2+} (*15*, *16*). Recently, Gagné et al. (*12*) also demonstrated that an N-domain that remains closed after Ca^{2+} binding has its affinity diminished 100-fold. Furthermore, from the structures solved by NMR, they calculated the accessible surface area of nonpolar groups. Considering only residues 4−88 of N-domain, Gagné et al. (*12*) observed the following values: 2866 Å^2 for apo N-domain and 3386 Å^2 for two Ca^{2+}−N-domain. In their study, the conformational change induced by Ca^{2+} involved an increase of 520 Å^2 in surface area exposed to solvent. This value is close to the gain in solvent-accessible surface area (571.5 ± 78.3 Å^2) calculated from the osmotic approach (Figure 8) shown here. Figure 10 sketches the closed-to-open transition of troponin C where hydration plays a crucial role. According to our data, 64 water molecules are in transit when the N-domain of TnC undergoes the conformational change involved in its opening.

As presented, the volume change of folding of N-domain in the Ca^{2+}-bound state (Table 2) is slightly higher than in the apo form (Table 1), which can be explained either by the presence of new electrostatic interactions and/or by an increase in the content of cavities when Ca^{2+} binds. Glycerol has practically no effect on the volume change of folding for the apo N-domain, whereas it decreases the ΔV of folding for the Ca^{2+}-bound state. This result may be explained by a decrease in the overall dynamics of the conformation of the Ca^{2+}-bound state in the presence of glycerol, which corroborates the data from the bis-ANS experiments showing that in the presence of glycerol the protein does not undergo the opening transition when it binds Ca^{2+}.

The thermodynamic data presented here also reinforce the idea that stability is inversely correlated to Ca^{2+} affinity. In Figure 11, we plot the values for free energy change of the apo forms (ΔG^{o}_{apo} as a function of pCa_{50}, using our own data. We also include the ΔG^{o}_{apo} for folding the N-domain at 1 °C (unpublished results). The data show a correlation between ΔG_{apo} and pCa_{50}. It is possible that there is a limit to this correlation, as stated by Grabarek et al. (*21*). The affinity of TnC for Ca^{2+} has to have a limit; otherwise, the ion would never leave the binding sites. If the inverse correlation between thermodynamic stability and Ca^{2+} affinity holds, we would expect the limit to be reached when

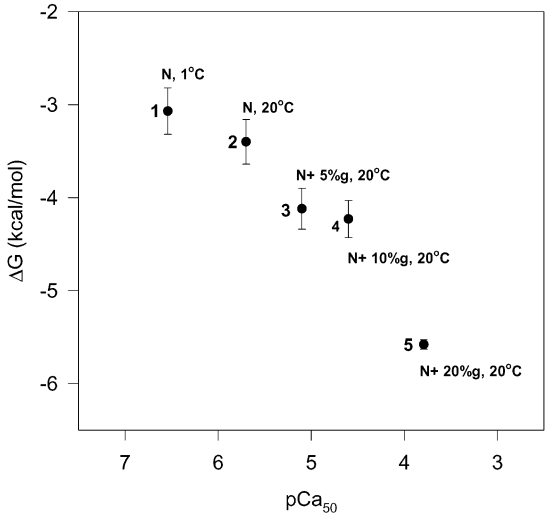

FIGURE 11: ΔG^{o}_{apo} (kcal/mol) versus pCa_{50}. Proteins: (1) F29W/N-domain, at 1 °C; (2) F29W/N-domain, at 20 °C; (3) F29W/N-domain plus 5% glycerol, at 20 °C; (4) F29W/N-domain plus 10% glycerol at 20 °C and (5) F29W/N-domain plus 20% glycerol, at 20 °C. Values of pCa_{50} and ΔG^{o}_{apo} (1) were extracted from Suarez et al. (unpublished results); the rest are from Tables 3 and 4.

a protein or a domain has a very poor thermodynamic stability and is extremely unstructured. The isolated C-domain would represent the limit of such a situation in which the Ca^{2+} affinity is very high and the stability is very low. It is noteworthy that this domain is bound to Mg^{2+} present at high concentrations inside cells most of the time. Our data would predict that when bound to Mg^{2+}, the C-domain would show a gain in structure and stability, which in fact occurs (*20*). Other Ca^{2+}-binding proteins such as parvalbumin and thermitase display Ca^{2+} affinities in the range of pCa 9−10. In the case of thermitase, Ca^{2+} binding is related to the stability of the protein, and no other physiological role has been attributed to this binding (*53*). Thus, this protein must be tightly bound to Ca^{2+} all the time. Indeed, it is possible that each Ca^{2+}-binding protein has its own "ΔG^{o}_{apo} versus pCa_{50} curve", rather than conforming to a single curve for all members of the family.

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