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Mimicry of the calcium-induced conformational state of troponin C by low temperature under pressure

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ABSTRACT **Calcium binding to the N-domain of troponin C initiates a series of conformational changes that lead to muscle contraction. Calcium binding provides the free energy for a hydrophobic region in the core of N-domain to assume a more open configuration. Fluorescence measurements on a tryptophan mutant (F29W) show that a similar conformational change occurs in the absence of Ca^{2+} when the temperature is lowered under pressure. The conformation induced by subzero temperatures binds the hydrophobic probe bis-aminonaphthalene sulfonate, and the tryptophan has the same fluorescence lifetime (7 ns) as in the Ca^{2+}-bound form. The decrease in volume ($\Delta V = -25.4$ ml/mol) corresponds to an increase in surface area. Thermodynamic measurements suggest an enthalpy-driven conformational change that leads to an intermediate with an exposed N-domain core and a high affinity for Ca^{2+}.**

Troponin C (TnC) is a Ca^{2+}-binding protein of the calmodulin superfamily that interacts specifically with troponin I in the troponin–tropomyosin regulatory complex on the thin filaments of vertebrate skeletal muscle (1–3). The crystal structures of avian TnC (4–6) reveal a dumbbell shape, with two globular domains linked by an extended α-helix. The N- and C-terminal domains are structurally homologous, each having two divalent-cation binding sites that consist of a helix-loop-helix motif in which 6 of 12 amino acid residues of the loop contribute to coordination of the cation. The C-domain has two high-affinity sites for Ca^{2+} that also bind Mg^{2+}, whereas the N-domain has two low-affinity, Ca^{2+}-specific sites (7). Each N-domain site is flanked by two α-helices (lettered A–D), and in addition there is an N-terminal helix closely apposed to helices A and D (Fig. 1*A*). Contraction is triggered when Ca^{2+} released from the sarcoplasmic reticulum binds to the low-affinity sites. Calcium binding promotes large conformational changes in the TnC structure (7). Recently, NMR data (8, 9) have suggested that Ca^{2+} binding to the low-affinity sites of TnC induces movement of helices B and C away from helices A, D, and N, confirming the general features of the model proposed by Herzberg *et al* (10). The hydrophobic cavity thus formed would favor association with TnI (7, 10, 11). Previous evidence for exposure of a hydrophobic patch has come from chemical cross-linking and fluorescence energy transfer studies (reviewed in ref. 7) as well as from changes in Ca^{2+} affinity in site-directed mutants designed to reduce or enhance (12–15) the energy barrier for exposure of the nonpolar side chains in the core of the N-domain.

Here we use fluorescence, pressure, and low temperatures to obtain quantitative thermodynamic data for the conformational change involving exposure of hydrophobic surfaces in the isolated N-domain. We describe a conformational intermediate induced by a physical trap (low temperature under pressure), one that mimics the conformation induced by Ca^{2+}. Hydrostatic pressure drives the structure of proteins to a thermodynamic state of smaller volume (for reviews, see refs. 16–20). Pressure has the advantage of allowing experiments at subzero temperatures to be performed in solution, since it lowers the freezing point of water (to −20°C at 2.0 kbar) (20–23). Both an increase in pressure and a decrease in temperature drive a protein in solution to a lower entropic state, one that favors binding of nonpolar amino acid residues to water (24, 25). Therefore, lowering the temperature under pressure serves to induce exposure of hydrophobic side chains, leading to protein conformational changes or even subunit dissociation and cold denaturation (20–23, 25, 26). The main point of this paper is to show that hydrostatic pressure and subzero temperatures change TnC/N-domain to a conformation similar to the Ca^{2+}-bound form, as determined by steady-state and dynamic fluorescence methods. Principally as a result of the decrease in temperature, a hydrophobic region is exposed in the N-domain in the Ca^{2+}-free form, similar to the conformational change promoted by binding of Ca^{2+}. The methodology we have used makes it possible to obtain thermodynamic data for the intermediate with the exposed core and to estimate the increase in surface area that accompanies exposure of the hydrophobic patch.

MATERIALS AND METHODS

The F29W/N-domain was prepared as previously described (27, 28). The buffer used in the pressure experiments was 100 mM Tris·HCl, 100 mM KCl, 1 mM DTT, 1.5 mM EGTA, and 0 or 2.1 mM $CaCl_2$, adjusted to pH 7.0 at 22°C. Final protein concentrations are given in the figure legends. Steady-state fluorescence was measured in an ISS/K2 spectrofluorimeter (ISS, Champaign, IL), with the excitation set at 280 nm and emission at 300–400 nm. For the pressure experiments, a pressure cell from ISS equipped with quartz windows was used (26). The extent of Ca^{2+} binding to TnC was calculated by using the fluorescence intensity F at 336 nm.

The equilibrium constant (K) at a given pressure was determined from the fractional changes in fluorescence intensity (γ), where $\gamma = (F - F_o)/(F_f - F)$, F_o being the intensity of the zero Ca^{2+} state, F_f the intensity of the Ca^{2+}-bound state (both at room temperature and atmospheric pressure), and $K = \gamma/(1 - \gamma)$. Thus, The final state is that of maximum fluorescence intensity, the Ca^{2+}-bound form. Since $-RlnK = \Delta G/T$, a plot of lnK against $1/T$ (a van't Hoff plot) yields enthalpy from the slope and entropy from the intercept on the ordinate axis. The van't Hoff plots were linear ($r > 0.99$).

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Abbreviations: TnC, troponin C; bis-ANS, bis-aminonaphthalene sulfonate.

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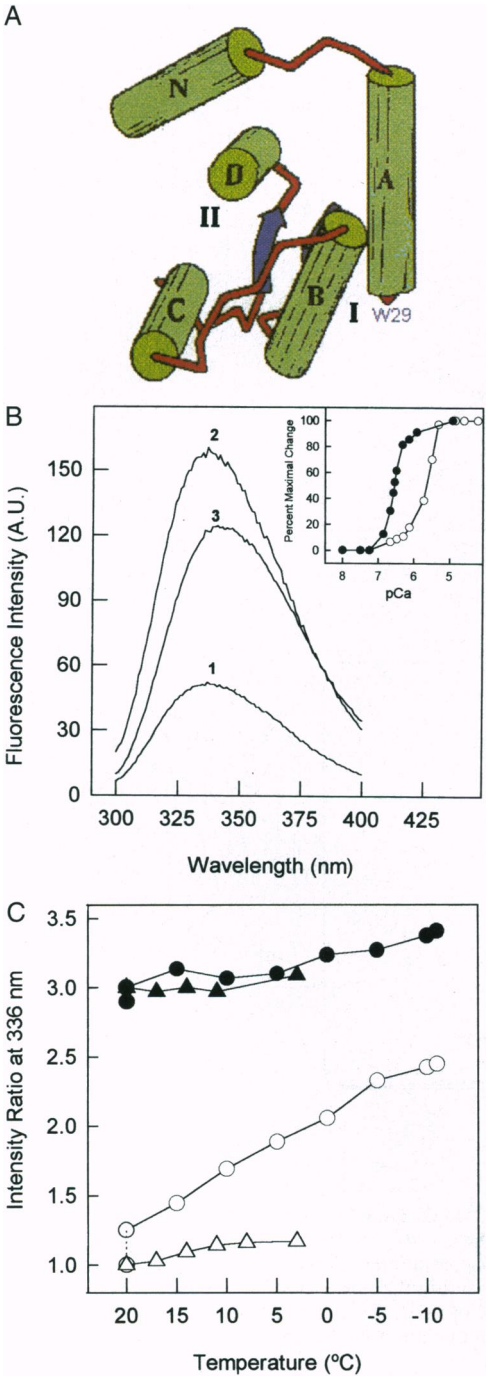

FIG. 1. (*A*) Structural model of TnC/N-domain in the absence of Ca^{2+}, showing the two Ca^{2+}-binding sites (I and II) and the position of the tryptophan (W29) introduced by mutagenesis at the junction between helix A and the first Ca^{2+}-binding site. Structure based on published coordinates for residues 1–90 (4). (*B*) Tryptophan emission spectra of F29W/N-domain under atmospheric pressure and 20°C in the absence (*1*) and in the presence of 0.6 mM free Ca^{2+} (*2*) and in the absence of Ca^{2+} at 2.2 kbar and −11°C (*3*). (*Inset*) Ca^{2+} titration curves at 20°C (○) and 1°C (●). The Hill coefficients for Ca^{2+} are 1.98 and 2.08, respectively, and the apparent dissociation constants (pCa_{50}) are 5.90 and 6.54. (*C*) Increase in the fluorescence intensity at 336 nm promoted by lowering the temperature under 2.2-kbar pressure in the absence (○) and in the presence (●) of Ca^{2+}. The triangles show the increase in fluorescence at 336 nm caused by a decrease in temperature at atmospheric pressure in the absence of Ca^{2+} (△) or in the presence (▲) of Ca^{2+}. The data are the ratios between the fluorescence intensity in each condition and the intensity in the absence of Ca^{2+} at atmospheric pressure and 20°C (empty symbol at lower left). The dashed lines between circles at 20°C show the small increase in fluorescence intensity that occurs when pressure is applied. In all cases the protein concentration was 5 μM.

Table 1. Spectroscopic properties of F29W-N domain of TnC in different conditions

	Atmospheric pressure, 20°C		2.2 kbar, −11°C
	EGTA	0.6 mM Ca^{2+}	EGTA
Intensity ratio at 336 nm	1	3.08	2.44
Ratio of bis-ANS fluorescence*	1	2.14	1.79
Lifetime†	c_1 = 2.87 ns f_1 = 0.98 χ^2 = 4.79	c_1 = 7.30 ns f_1 = 0.97 χ^2 = 1.27	c_1 = 7.20 ns f_1 = 0.898 χ^2 = 5.56

*The bis-aminonaphthalene sulfonate (bis-ANS) binding experiments were done in the presence of 5 μM of the probe. The samples were excited at 360 nm and emission collected at 400-600 nm.

†Center (c) and fraction (f) of the principal Lorentzian component of lifetime distribution. In each case a small discrete component (f_2 = 0.02 − 0.10) was also present (see Fig. 2*C*).

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 Ca–EGTA complex. The solutions contained 50 mM Mops, 100 mM KCl, 1 mM DTT, and 1.0 mM EGTA. The pH was adjusted to 7.0 at 1 and 20°C.

Lifetime measurements were performed in a multifrequency cross-correlation phase and modulation fluorometer (ISS/K2), as described (29–32). Samples were excited at 295 nm with a 300 W xenon lamp and emission was collected using 7-54 and 0-52 filters. For pressure experiments, light scattered from ficol particles was used as reference (32). The choice of fitting with Lorentzian and discrete components was based on χ^2 values (Table 1) and plots of weighted residuals.

RESULTS AND DISCUSSION

TnC from chicken skeletal muscle has no tryptophan or tyrosine in its primary sequence. Recently, mutants of whole TnC and of its isolated N- and C-domains have been constructed using site-directed mutagenesis to replace phenylalanine residues with tryptophan (15, 27, 28). These mutants have been used extensively for fluorescence investigation (27, 28). Calcium binding to F29W/N-domain (residues 1–90) promotes a large increase in fluorescence (Fig. 1*B*), indicating that Trp-29 now samples an environment where some internal quenching is relieved. A similar increase in fluorescence is induced by lowering the temperature to −11°C under pressure (2.2 kbar = 220 MPa) in the absence of Ca^{2+} (Fig. 1*B*). The fluorescence increases to nearly the same extent as when Ca^{2+} is present at room temperature and atmospheric pressure (Table 1). In general, hydrophobic interactions in proteins are weakened by a decrease in temperature (21–23, 25, 33); thus we suggest that the low temperature has weakened internal interactions within a hydrophobic region of the N-domain. A stepwise decrease in temperature in the absence of Ca^{2+} promotes a progressive increase in tryptophan fluorescence (Fig. 1*C*). In the presence of 0.6 mM free Ca^{2+}, the fluorescence intensity is already high, and there is little further increase as the temperature is lowered to −11°C. This result leads to the hypothesis that low temperature drives the N-domain toward the Ca^{2+}-bound conformation. The increase in emission is completely reversible when temperature and pressure are returned to initial conditions (not shown). When temperature alone is decreased at atmospheric pressure, the changes in intensity are small, with or without Ca^{2+} (triangles in Fig. 1*C*). Titration of the fluorescence change caused by

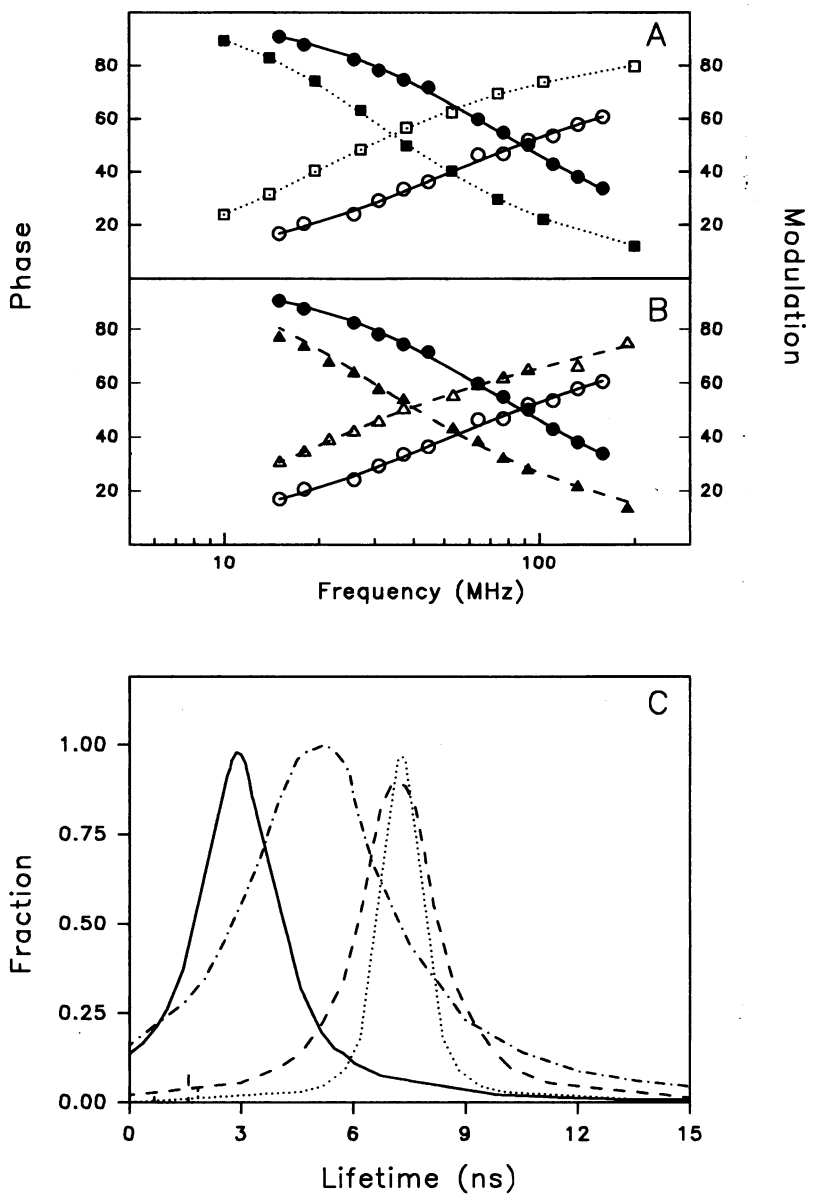

FIG. 2. (*A* and *B*) Phase shift (open symbols) and modulation (filled symbols) of F29W/N-domain in different conditions. (*A*) At atmospheric pressure and 20°C in the absence of Ca^{2+} (●, ○) and in the presence of 0.6 mM Ca^{2+} (■, □). (*B*) At atmospheric pressure and 20°C in the absence of Ca^{2+} (●, ○) and in the absence of Ca^{2+} at 2.2 kbar and −11°C (▲, ○). The protein concentration was 20 μM. (*C*) Lorentzian lifetime distribution of F29W/N-domain at atmospheric pressure and 20°C in the absence of Ca^{2+} (——) and in the presence of 0.6 mM Ca^{2+} (···); at 2.2 kbar and 0°C in the absence of Ca^{2+} (–·–·–) and at 2.2 kbar and −11°C (– – –) in the absence of Ca^{2+}. Each data point represents the average of seven measurements, and the error is less than 0.2 degree for the phase and 0.004 for the modulation measurements.

Ca^{2+} at atmospheric pressure is displaced to higher pCa values at 1°C (Fig. 1*B Inset*). This result shows that lowering the temperature increases the apparent affinity for Ca^{2+}, as expected for a reaction that is favored by a decrease in entropy.

The thermodynamic parameters at 2.2 kbar were determined from a van't Hoff plot of the data in Fig. 1*C* as described in *Materials and Methods*. Both enthalpy and entropy changes were negative ($\Delta H = -20.08 \pm 0.79$ kcal/mol; $\Delta S = -71.79 \pm 2.73$ cal/mol·K). These parameters were determined at different pressures and extrapolated to atmospheric pressure, revealing a favorable enthalpy (−31.04 kcal/mol) and an unfavorable entropy change (−111.0 cal/mol·K). The lower the pressure, the lower the temperature required to achieve the same extent of reaction. The net result at 25°C and 1 bar is a small positive value for ΔG (2.04 kcal/mol). The same set of data were fit using the equation that describes changes in free energy with pressure ($d(\Delta G)_T/dp = \Delta V$) to calculate ΔV, the standard volume change at atmospheric pressure that accompanies the conformational change reported by Trp-29 fluorescence. The obtained decrease in volume ($\Delta V = -25.4 \pm 3.2$ ml/mol) corresponds to an increase in nonpolar surface area exposed to the aqueous medium, as predicted in the model of Herzberg *et al.* (10) and recently suggested using NMR (9). The volume decreases when the hydrophobic surface is exposed because the protein–water interactions occupy less volume than the protein–protein interactions (16–20).

To corroborate the hypothesis that low temperature drives the N-domain toward the Ca^{2+}-bound conformation, we performed experiments with the hydrophobic probe, bis-ANS. In solution, this probe has a negligible fluorescence that increases dramatically upon binding to hydrophobic segments of proteins (34). A large increase in emission associated with bis-ANS binding to F29W/N-domain is promoted by Ca^{2+} binding or by lowering the temperature to −11°C at 2.2 kbar in the absence of Ca^{2+} (Table 1). These data show that both the decrease in temperature and the binding of Ca^{2+} lead to exposure of a hydrophobic region.

Time-resolved fluorescence measurements allow probing of the tryptophan environment in the nanosecond time frame, providing a "dynamic fingerprint" of the conformational state (29–32). Fluorescence lifetime measurements were performed to compare the excited state of the single tryptophan residue of F29W/N-domain in three different conditions: Ca^{2+}-free, Ca^{2+}-bound and in the absence of Ca^{2+} at low temperature under pressure (Fig. 2 *A* and *B*). The shorter lifetimes of the Ca^{2+}-free form required higher frequencies for adequate sampling of the phase shift and demodulation between excitation and emission, whereas the longer lifetimes of the Ca^{2+}-bound form as well as the low-temperature configuration were assessed by lower frequencies. In the absence of Ca^{2+}, the best fit for the data is achieved by a Lorentzian distribution of lifetimes centered at 2.87 ns, with a very small discrete component (Fig. 2*C*). In the presence of Ca^{2+}, as well as at low temperature under pressure, the larger component shifts to a longer lifetime (Fig. 2*C* and Table 1). This shift, an increase of 2.5-fold, indicates a longer-lasting excited state and is consistent with the large increase in tryptophan emission that takes place under these conditions (see Fig. 1*B*). The lifetime values found for the Ca^{2+}-bound form and for the conformation induced by low temperature at high pressure are identical, within experimental error (Table 1). This correlation suggests that Trp-29 experiences the same environment in the N-domain occupied by Ca^{2+} as in the N-domain configuration induced by low temperature in the absence of metal ion.

The lifetime distribution for TnC at an intermediate stage (2.2 kbar and 0°C—about 50% of the maximal change according to Fig. 1*C*) is included in Fig. 2*C*. The center of lifetime is 5.07 ns, in between 2.8 and 7.2 ns. However, the distribution was much broader, indicating that the rate of interconversion between the two forms lies in the same time window of the lifetime of the tryptophan.

Taken together, the four lines of evidence (fluorescence intensity changes, Ca^{2+}-binding curves, Trp lifetimes, and bis-ANS binding) may indicate the existence of an intermediate species of the TnC/N-domain that mimics the Ca^{2+}-bound state. This intermediate would be stabilized by the binding of Ca^{2+}:

$$\text{TnC/N-domain} \underset{1}{\Leftrightarrow} \text{TnC/N-domain}^* \overset{2\,Ca^{++}}{\underset{2}{\Leftrightarrow}} \text{TnC/N-domain}^*\,Ca_2$$

where the * species represents a conformation that has the nonpolar segment exposed to the aqueous environment and a high affinity for Ca^{2+}. Although the conformation that appears at 2.2 kbar and −11°C is clearly less disordered than at atmospheric pressure and room temperature, it is not completely denatured. The energy of emission of TnC decreases 150 cm^{-1} when the temperature is lowered to −11°C under 2.2 kbar, whereas it decreases by about 600 cm^{-1} when TnC is denatured by a high concentration of urea (not shown). The fluorescence intensity increases 2.5-fold in the low temperature/pressure condition, whereas it decreases when the protein is denatured by urea. The Trp emission of TnC in the presence of Ca^{2+} undergoes practically no change (<50 cm^{-1}) when the temperature is lowered under pressure. Although the Trp emission of TnC undergoes some red shift, which may indicate partial denaturation, when cooled under pressure in the absence of Ca^{2+}, it too is much less than the shift induced by complete denaturation provoked by urea.

The decrease in volume (25.4 ml/mol) in step 1 is related to an increase in exposed surface area (ΔS). Any volume change in a protein can be separated into two components:

$$\Delta V_t = \Delta V_c + \Delta V_l \quad [1]$$

where ΔV_c is the decrease in volume due to the disappearance of protein cavities and ΔV_l is the decrease in volume due to the replacement of protein-protein interactions by protein–solvent interactions. This component will be equal to $\Delta S \Delta l$, where Δl is the linear contraction due to the solvent constriction effect. In the case of a small conformational change, $\Delta V_c \ll \Delta V_l$, and therefore:

$$\Delta V_t \cong \Delta S\ \Delta l \quad [2]$$

When ΔV_t is induced by pressure, a rough estimate of ΔS can be obtained by assuming Δl approximates the isotropic linear contraction (L) under pressure as determined according to Bridgman (36, 37):

$$L = 1 - (V_p)^{1/3} \quad [3]$$

where V_p is the unit volume that results when the pressure is increased from 1 atm to 2.0 kbar. For hexane, each Å^3 decreases to 0.87 Å^3 and L is 0.0454 Å (36). Dividing the decrease in volume obtained experimentally for TnC/N-domain (25.4 ml/mol = 42.2 Å^3 per molecule) by 0.0454 Å gives a calculated exposed area of 929 Å^2. An upper limit can be calculated by using as a model a dipolar solvent such as water instead of hexane; in this case, the exposed area is 1648 Å^2. Taking into consideration that several approximations are involved, notably that $\Delta l = L$ and $\Delta V_c \approx 0$, an increase in surface area in the range of 900-1600 Å^2 is consistent with the associated change in enthalpy (−31 kcal/mol), and can be compared with the average values of −25 kcal per 1000 Å^2 of intersubunit surface exposure for the dissociation of oligomeric proteins (38). The net increase in surface area exposed by Ca^{2+} binding in the model of Herzberg *et al.* (10) is ≈400 Å^2, based on a partial listing of the exposed residues of the N-domain.

The low-temperature experiments under pressure have allowed us to measure the thermodynamic parameters related to step 1, the conformational change that precedes Ca^{2+} binding. The values for the overall reaction are those measured calorimetrically by Potter *et al.* (35), who showed that the reaction TnC ⇔ TnC*·Ca_2 occurs with a negative ΔG derived from a large negative enthalpy change (ΔH = −15.4 kcal/mol for the 2 Ca^{2+}-specific sites) and a small change in entropy (ΔS = 16 cal/mol·K). The changes in entropy and enthalpy and the resulting free energy changes that occur as the protein moves through the three states are represented in Fig. 3. In contrast to the overall reaction, the free-energy change (ΔG) for step 1 (TnC ⇔ TnC*) is a small positive value. Binding of Ca^{2+} in step 2 overcomes this small free-energy barrier, leading to different thermodynamic potentials (H, S, and G) for TnC*·Ca_2. Step 2 involves a large increase in entropy; together

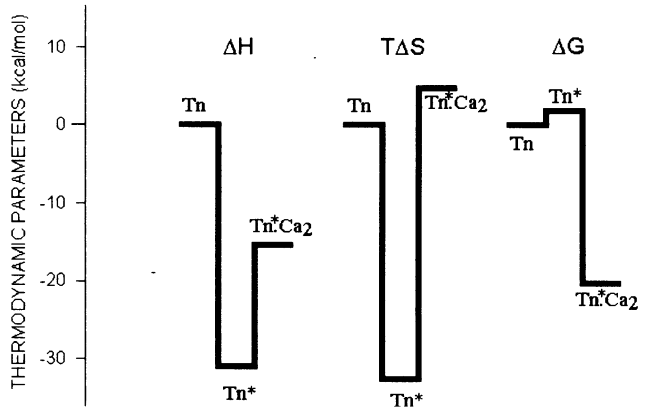

FIG. 3. Thermodynamic parameters for the conformational transition cycle that takes place as the N-domain structure opens and Ca^{2+} is bound. Each panel shows the values attributed to the three species of Tn that are formed as the reaction proceeds through steps 1 and 2 in the equation given in the text. The parameters associated with Tn*·Ca_2 species are from Potter *et al.* (35).

with the small increase in enthalpy, the final result is a favorable free energy change of −20.2 kcal/mol (Fig. 3), so that the overall reaction occurs spontaneously.

CONCLUSIONS

Our study presents clear-cut evidence for the exposure of a nonpolar surface previously postulated to take place in the N-domain of TnC when Ca^{2+} is bound (9, 10). The data we have presented show that exposure of a hydrophobic patch to the aqueous environment (formation of TnC*) is an enthalpy-driven process that is accompanied by a large decrease in entropy. This result indicates that TnC sacrifices hydrophobic stability to achieve function, i.e., it has to create a TnC (N-domain)–TnI (C-domain) contact region to activate the thin filament. The approach we have used is likely to be applicable to other biological switches that control cellular processes.

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