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The Regulatory Domain of Troponin C: To Be Flexible or Not To Be Flexible

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Abstract: The calcium-induced structural changes in the skeletal muscle regulatory protein troponin C (NTnC) involve a transition from a 'closed' to an 'open' structure with the concomitant exposure of a large hydrophobic interaction site for target proteins. Structural studies have served to define this conformational change and elucidate the mechanism of the linkage between calcium binding and the induced structural changes. There are now several structures of NTnC available from both NMR and X-ray crystallography. Comparison of the calcium bound structures reveals differences in the level of opening. We have considered the concept of a flexible open state of NTnC as a possible explanation for this apparent discrepancy. We also present simulations of the closed-to-open transition which are in agreement with the flexibility concept and with experimental energetics data.

INTRODUCTION

NMR and X-ray crystallography are powerful structural techniques, and the number of protein structures solved by these methods increases every years. Although structures provide a generous amount of information on a particular system, an important aspect is often neglected: dynamics. Even in a family of NMR structures, true flexibility information can rarely be extracted. It is however important to characterize the dynamics of a system, as this can provide information on energetics and on populated conformations which might not be seen in a static structure.

There are several aspects of dynamics which can be of interest for function. This paper focuses on large scale motions, i.e. motion of whole helices. The system studied here is the N-terminal domain of troponin C (NTnC), a domain which regulates muscle contraction thru a

large calcium-induced structural change.

Troponin C

Transient increases in intracellular calcium concentration in striated muscle cells are recognized by the thin filament proteins to regulate muscle contraction. This cascade of events is initiated by calcium binding to TnC. The resultant signal is transmitted to the other members of the thin filament (troponin I, troponin T, tropomyosin, and actin), which in turn modifies the interaction between the thick and thin filaments, leading to muscle contraction.[1-5]

Skeletal TnC is an 18 kD protein and is a member of the family of the EF-hand calcium binding proteins.[6] TnC comprise four EF-hand helix-loop-helix motifs as calcium binding sites (I-IV). Sites I and II are paired as a unit in the N-terminal half and sites III and IV form another pair in the C-terminal half of the molecule. Sites III and IV bind calcium ($K_{Ca} \sim 10^7\ M^{-1}$) with high affinity and are believed to be always occupied by either calcium or magnesium ($K_{Mg} \sim 10^3\ M^{-1}$) under physiological conditions for a structural role, whereas calcium binds to sites I and II with lower affinity ($K_{Ca} \sim 10^5\ M^{-1}$) and regulates muscle contraction.[7, 8] The structural and functional properties of TnC have been extensively reviewed in the recent years.[1-5, 9, 10]

Structures and calcium-induced structural change of skeletal NTnC

A large amount of structural information on the regulatory domain of skeletal TnC is now available: X-ray crystal structures of whole TnC where NTnC is in the apo state,[11, 12] an early proposed model of the calcium state of NTnC,[13] NMR solution structures of NTnC in both the apo and calcium states,[14] NMR solution structure of whole TnC where NTnC is in the calcium state,[15] NMR solution structure of an E41A mutant of NTnC in the calcium form,[16] an X-ray crystal structure of NTnC in the calcium form,[17] and X-ray crystal structures of whole TnC where NTnC is in the calcium state.[18]

These structures have defined the calcium-induced structural changes in TnC, and elucidated the mechanism of the linkage between calcium-binding and the concomitant conformational changes. The structural transition in the regulatory domain of TnC on calcium-binding involves an opening of the domain through a large change in interhelical angle, leading to the increased exposure (~ 500 $Å^2$) of an extensive hydrophobic patch.[14] The magnitude of the movement of helix-B and helix-C is characterized by a large change in interhelical distance, resulting in displacements of 12 Å for the C-terminal end of helix-B, 16 Å for the N-terminal end of helix-C, and 19 Å for the center of the B-C linker. The calcium induced opening of NTnC is represented in figure 1. The calcium induced structural change can be quantified in terms of the interhelical angles between helix-A and helix-B, and between helix-C and helix-D; these angles are listed in Table 1 for the various structures of NTnC.

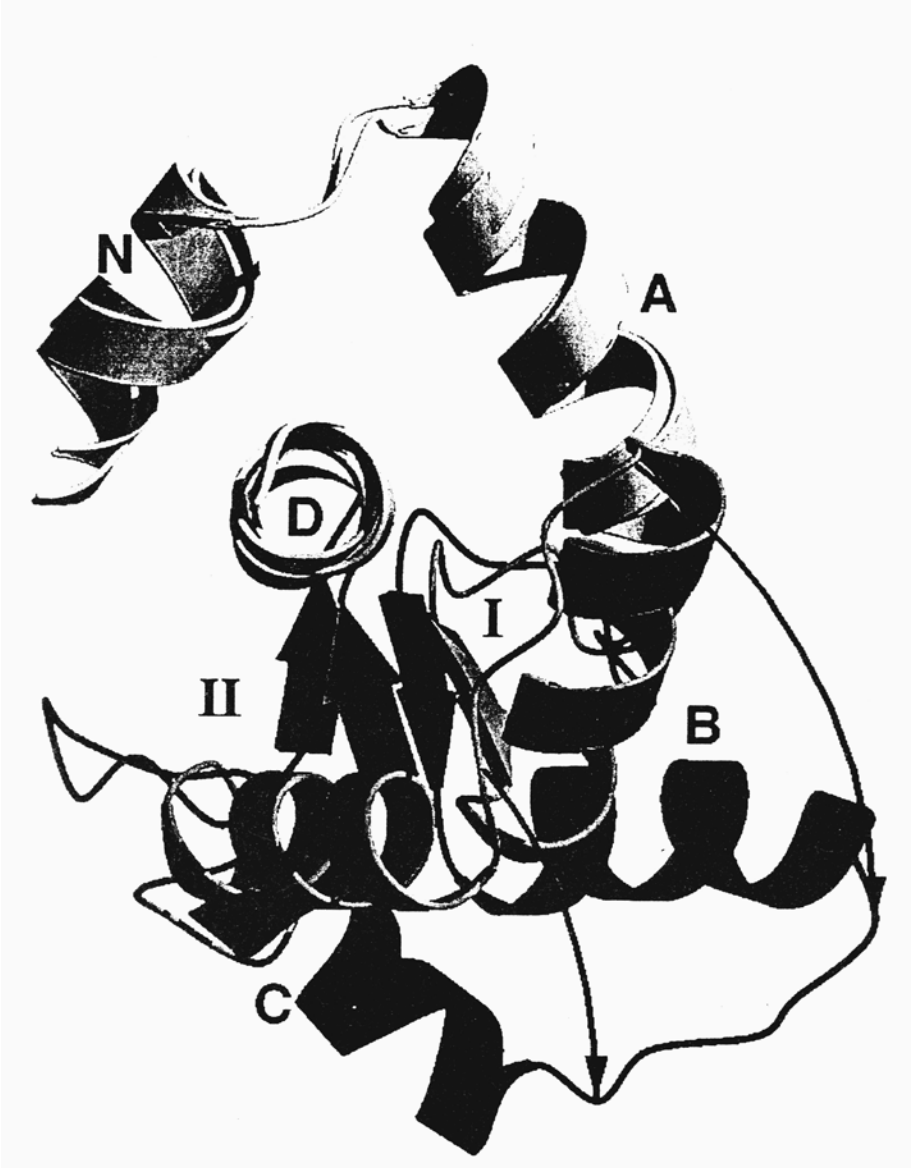

Fig. 1. Representation of the calcium induced opening of the NTnC.[14] The apo form[11] and the Ca_2 form[14] were superimposed using residues in helices N, A, and D (shown in light gray). The movement of the helix-B/helix-C unit (shown in medium gray for the apo form and in dark gray for the Ca_2 form) upon calcium binding is indicated by the arrows.

In order to obtain insight into the mechanism of regulation within TnC, that is, the coupling between calcium binding and subsequent structural change, we solved the structure of

Table 1. Interhelical angles[a] in the N-terminal domain of troponin C (NTnC)

Structure	Interhelical angle	
	A/B	C/D
NTnC, apo		
X-RAY[b]	139	146
NMR, 30 °C[c]	127 ± 3 (120-135)	124 ± 4 (115-132)
NMR, 4 °C[d]	134 ± 4 (124-148)	156 ± 3 (147-172)
NTnC, Ca_2		
NMR[e]	80 ± 6 (63-100)	71 ± 8 (47-97)
X-RAY 1[f]	97	83
X-RAY 2[g]	104	97
X-RAY 3[h]	95	89
E41A-NTnC, Ca_2		
NMR[I]	131 ± 4 (124-138)	131 ± 5 (120-138)

[a] The axis orientation for an α-helix was defined by two points, where the first point is the average of the first 11 backbone atoms and the second point is the average of the last 11 backbone atoms. The program used to calculate the angle was IHA (S.M. Gagné) and is available from the authors. The start and end of the helices were (using skeletal chicken TnC numbering): A, 16-28; B, 42-48; C, 55-64; D, 75-85;.

[b] PDB entry code 4TNC and 5TNC. [c] PDB entry code 1TNP. [d] PDB entry code 1SKT. Recalculated 200 structures with XPLOR. [e] PDB entry code 1TNQ. Recalculated 1000 structures with XPLOR. [f] PDB entry code 1AVS. [g] PDB entry code 1TN4 and 2TN4. [h] PDB entry code 1TCF. [i] PDB entry code 1SMG.

a calcium-bound mutant of NTnC (E41A-NTnC).[16] In this mutant, the bidentate ligand to the calcium in site I (E41) was removed. Our initial goal was to explore the contribution of site I into the calcium induced structural change by making site I defunct. The calcium titration of E41A-NTnC revealed that despite removal of the bidentate ligand, site I was still able to bind calcium although with a diminished affinity (K_d = 1-2 mM).[19]

The structure of E41A-NTnC remains partially closed upon calcium binding, indicating that the linkage between calcium-binding and the induced conformational change has been broken. This provides a snapshot of NTnC between the off and the on state. Comparison of the helix packing in E41A-NTnC with other NTnC structures reveals valuable information. The interhelical angles of calcium bound E41A-NTnC are more similar to the apo forms rather than the Ca_2 form (Table 1). Therefore, the hydrophobic patch is not completely exposed upon calcium-binding unless the bidentate ligand in site I (E41) is involved into calcium-coordination. Although several factors contribute to the triggering mechanism, the opening of the NTnC structure is ultimately dependent, in a temporal and energy balance sense, on one amino acid (E41)[16].

***To be flexible or not to be flexible? That is the question*!**

A general feature emerges when we compare the calcium bound NTnC structures obtained by NMR and X-ray. First, the average NMR structure is more open than the X-ray structures. Second, the X-ray structures cover a range of interhelical angles. We explored the causes and possible implications related to this finding.

We must say that defining the interhelical orientation in the open form is difficult using NMR, at least when using standard NMR structure determination approaches. The reason for this is that the open structure has a very limited number of interhelical contacts shorter than 5Å, and therefore the number of observed long range interhelical NOEs is modest. This is the principal reason why the interhelical angles cover a range of 15-20° in most NMR structures of TnC. Despite the fact that the opening is not well defined in the NMR structures, this does not necessarily mean they are inconsistent with the X-ray structure. When comparing the range of interhelical angles which is observed in the NMR structures with the crystal structure, one finds that the X-ray structures are in the range covered by the NMR family of structures, usually at the most closed extremity. This is best illustrated for the C/D interhelical angle of the calcium bound N-domain of TnC. Figure 2 compares the distribution of this interhelical angle in the NMR structures with the corresponding X-ray structures. This figure is consistent with a flexible open state where the crystal structures would correspond to the most compact open form.

Although we cannot directly interpret the lack of definition in the NMR structures as true

flexibility, the question still exists; is the open form flexible in terms of interhelical arrangement? The NOE data would not be significantly different whether the open form is rigid or covers a range of 15-20° in interhelical angles. Thus NMR structural data has not yet provided an answer to the question in this case, and we are presently investigating NMR relaxation approaches. Interestingly, the various X-ray structures which have been solved for calcium bound NTnC support the concept of a flexible open form. As can be seen in Figure 2, the C-D interhelical angle varies significantly amongst the crystal structures.

The notion of a flexible open state is actually not without sense. The major determinant which keeps helices packed together in globular proteins is the favorable interactions between the helices (usually involving hydrophobic amino acids). In the open form of the NTnC, the A/B and C/D helices do not make favorable contacts for the most part. Therefore the limited number of interhelical interaction is likely too small to hold these helices together. Flexibility may also be desirable for function.[20]

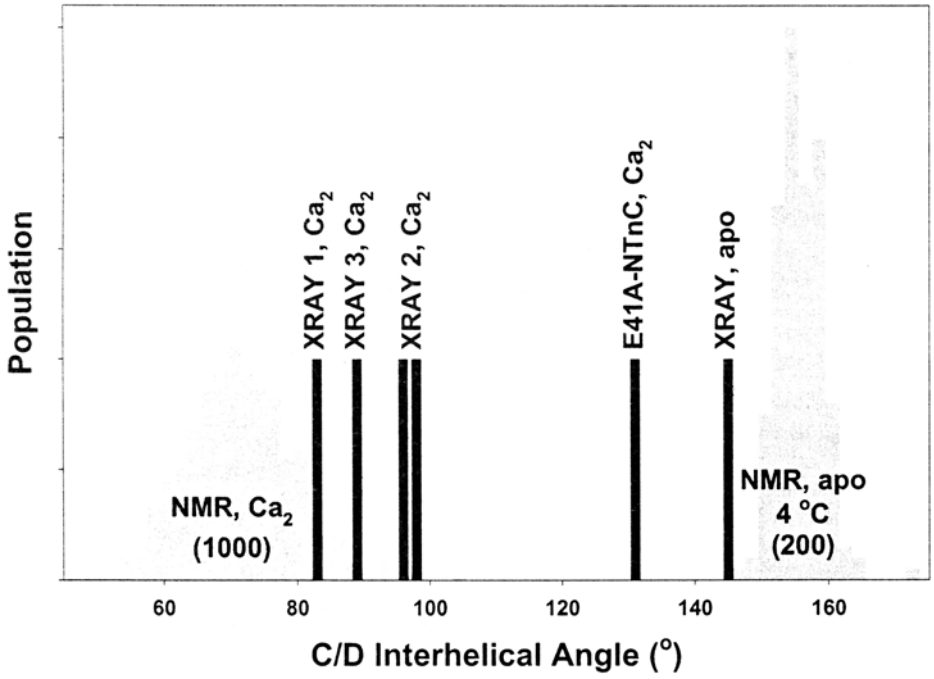

Fig. 2. Distribution of the C/D interhelical angle in the structures of NTnC. The labeling corresponds to the structures listed in Table 1.

It is interesting to note that TnC was not really made to be in an open state with an empty hydrophobic patch. In fact, the population of the empty open state is most probably insignificant in muscle. TnI, being already located just a few angstroms away from the regulatory domain of TnC, is readily available for binding to the hydrophobic patch once the calcium induced conformation change occurs.

The closed-open transition

Questions about the flexibility of the calcium bound open state of NTnC have led us to consider the opening and closing motion as a whole (i.e. from fully closed to fully open), not just fluctuations within the open state. We believe that the opening and closing motion of NTnC occurs via a hinge-bending motion, and that the full range of motion occurs even in the absence of calcium binding. This assumption is reasonable since the structure of NTnC consists roughly of two lobes connected by a flexible loop region. The hinge-bending motion has been studied extensively in the literature,[21] and theoretical studies on such systems date as early as 20 years ago.[22]

Before proceeding with theoretical calculations we assessed if the opening and closing of NTnC could indeed occur via a hinge-bending motion. We obtained the free software program "DynDom" (http://rugmd0.chem.rug.nl/~steve/DynDom/dyndom.home.html), which is designed specifically to find possible hinges in a protein given two conformations. The results of supplying the program with an open and a closed structure of NTnC clearly indicated that there was a hinge in the flexible loop regions, exactly where we had surmised it to be.[16] The structures we supplied to the program consisted of residues 7-87 of one chain of the calcium bound structure (PDB entry code 1AVS)[17] and residues 7-87 of the apo structure (PDB entry code 5TNC).[11]

We then attempted to determine a free energy profile of the opening and closing of NTnC. We used the Experimental Protein Morphing Server (EPMS) (http://bofur.csb.yale.edu/~protmot) which is available for free use. Given any two structures, the EPMS will "morph" one into the other, generating a series of intermediate structures. This technique does not rely on the assumption that the motion occurs via a hinge-bending mode, but the structural homology between the rigid domains in the open and closed states is such that the morphing approximates a hinge-bending motion well. Initially we supplied the EPMS with the two structures previously supplied to the program DynDom, and generated a series of 10 structures ranging from the fully closed to the fully open state. However, even though such a morphing represents a plausible pathway for the opening and closing, there is no way of ensuring that this is indeed the reaction coordinate of the opening. The reaction coordinate of such a motion is by no means obvious, and extensive work has been performed to determine the reaction

coordinate of similar motions.[23] To obtain a second possible reaction coordinate we supplied the EPMS with a series of three structures. The first two were the same open and closed structures previously supplied, and the third consisted of the calcium bound E41A-NTnC structure discussed previously. The calcium bound E41A-NTnC structure was supplied as an intermediate in the opening reaction, and thereby forced the reaction coordinate to proceed through that structure. This yielded a series of 19 structures ranging from fully closed to fully open.

The two series of structures allowed for the calculations of the free energy of opening over each reaction coordinate. The calculations were done using the program Structural Thermodynamic Calculations (STC) developed in our lab, which is based on a method described in the literature.[24] The results are summarized in Figure 3, using the fully closed structure as the reference point for the energy calculations. Of particular interest is the net

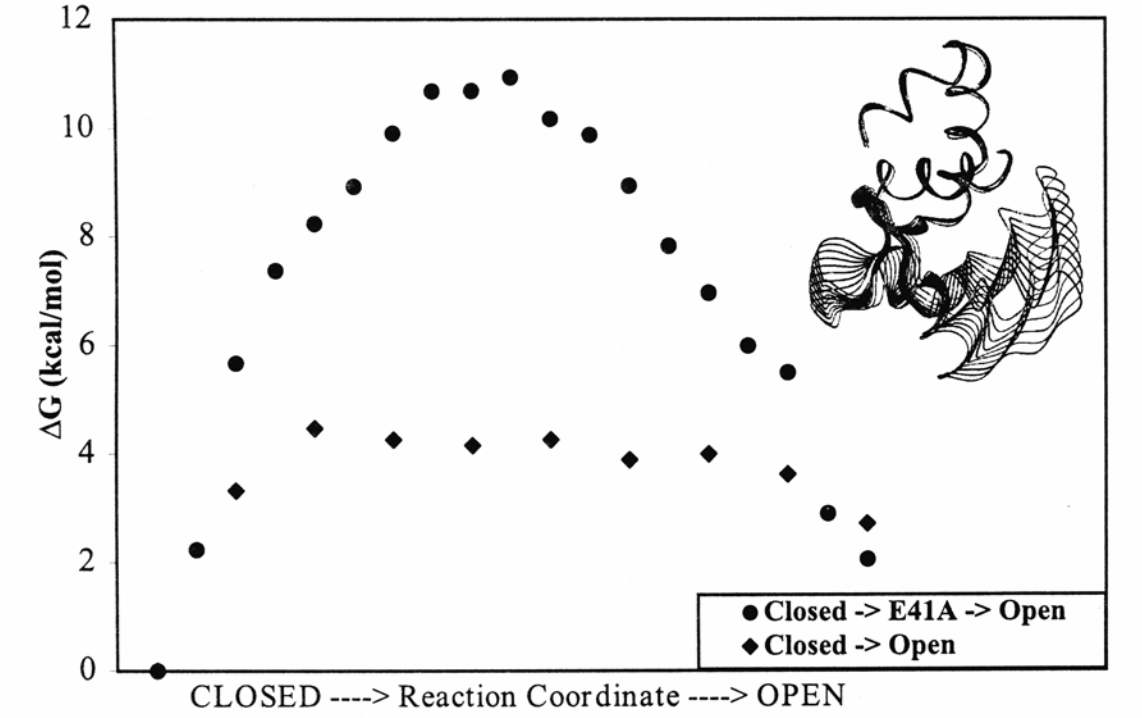

Fig. 3. Graph showing the STC results for the free energy of opening of apo NTnC. The results from two series of morphed structures are displayed: (_) morphed directly from the closed to the open state, (ℓ) morphed first to the E41A-NTnC structure, and then to the open structure. Inset: diagram of 10 pdb files generated by the Morph Server (_).

energy change from the fully closed to the fully open state. Without E41A-NTnC in the reaction coordinate the ΔG_{net} = 2.708 kcal/mol, and with E41A-NTnC in the reaction coordinate the ΔG_{net} = 2.048 kcal/mol. These figures indicate that 1% or 3% respectively of NTnC molecules in the absence of calcium exist in the open state. The numbers are also very close to a previously suggested value of ΔG_{net} = 2.04 kcal/mol.[25] A second key feature is the difference in barrier heights between the two reaction coordinates, on the order of 4.5 kcal/mol for the pathway without the E41A-NTnC structure, and on the order of 11 kcal/mol for the pathway with the E41A-NTnC structure. This indicates that if both pathways are possible, and that the energetics as calculated are the main contributing factor, then the pathway without the E41A structure is much more favorable. This can not however be used to define the reaction coordinate because it is unclear what other factors which might contribute. Clearly though, there is a large difference to be obtained between the different reaction coordinates, and thus the pathway of the opening is a crucial piece of information to understanding the system.

These results are a promising start, but only touch on the surface of the opening and closing process. There is clearly more work to be done in defining the reaction coordinate in order to ensure that the free energy curves calculated are accurate. Further, to provide a comprehensive picture of the system, similar calculations need to be performed for NTnC in the calcium bound state. Unfortunately, as of now we are unable to include calcium ions in our STC calculations.

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