FEBS Letters 347 (1994) 152–156

FEBS 14140

The presence but not the sequence of the N-terminal peptide in cardiac TnC is important for function

Wen Liu, Darrell G. Dotson, Xin Lin, James J. Mullen III, Manuel L. Gonzalez-Garay, Qingxian Lu, John A. Putkey*

Department of Biochemistry and Molecular Biology, The University of Texas Medical School, 6431 Fannin, Houston, TX 77030, USA

Received 20 April 1994; revised version received 11 May 1994

Abstract

The most diverged region of the primary amino acid sequence between cardiac (cTnC) and fast skeletal troponin C is the N-terminal ten amino acids. We report here that major changes in the primary sequence of this region in cTnC had a minimal effect on the ability of the mutant proteins to recover maximal activity in TnC-extracted cardiac and fast skeletal muscle myofibrils. However, deletion of the N-terminal nine amino acids resulted in a 60% decrease in maximal Ca^{2+}-dependent ATPase activity with only a small change in the pCa_{50} of activation. Deletion of the N-terminal peptide did not appear to appreciably affect the Ca^{2+}-binding properties of cTnC, but it did alter the interaction with hydrophobic fluorescent probes. Thus, the presence but not the sequence, of the N-terminal extension is important for the maximal activity of cTnC. The N-terminal helix may function in a relatively non-specific manner to prevent unfavorable interactions between domains in cTnC or between cTnC and other troponin subunits.

Key words: Cardiac troponin C; Muscle regulation; Mutagenesis

1. Introduction

The N-terminal peptide of troponin C (TnC), defined as those amino acids that are N-terminal to helix A, is the most diverged region between the cardiac (cTnC) and skeletal (sTnC) isoforms [1,2]. Calmodulin (CaM) shares a high degree of structural similarity with TnC [3–6] and can functionally substitute for cTnC and sTnC in the corresponding muscle types in vitro [7,8], yet it lacks and N-terminal peptide. These comparisons suggest that the N-terminal peptide of TnC is not essential for activity, but that it might confer isoform-specific functional differences to cardiac and skeletal TnC.

In the study reported here, we have tested the functional significance of the N-terminal peptide in cTnC by substitution and deletion mutagenesis followed by functional characterization in cardiac and fast skeletal myofibrils. The data demonstrate that the presence but not the primary sequence of the N-terminal peptide in cTnC is important for function.

2. Materials and methods

The construction of expression plasmids for the recombinant proteins cTnC3 and cTnCN1 was described previously [9]. Expression plasmids for cTnCN3 and cTnC(ΔN) were prepared by replacing the *Bam*HI–*Hin*cII restriction fragment from the plasmid pMC6B with various synthetic double-stranded DNA fragments. This resulted in either deletion or modification of the first 11 codons of the chicken cTnC cDNA. The modified cDNAs were isolated as *Nco*I–*Hin*dIII restriction fragments and used to replace the corresponding fragment in the expression plasmid pTnCPL3 which encodes normal cTnC [9]. The expression plasmid for cTnC(FLAG) was constructed using PCR.

All proteins were isolated using the chromatographic procedure described previously [9] with slight modifications. Western blots of cTnC(FLAG) proteins were performed according to standard procedures using a kit obtained from IBI/Kodak. Purified proteins were diluted with 0.05 M Tris, pH 7.4, 0.15 M NaCl and 0.1 ml was applied to Nytran nitrocellulose membranes. The membranes were allowed to dry, saturated with excess casein protein and exposed to the M1 or M2 FLAG antibody (IBI/Kodak) followed by an anti-rabbit antibody conjugated to horseradish peroxidase.

Myofibril ATPase assays were performed as described previously [10]. Equilibrium dialysis for the determination of Ca^{2+} binding capacity of the various proteins was performed using microdialysis chambers (BRL) in a buffer containing 50 mM MOPS, pH 7.0, 150 mM KCl, 1 mM dithiothreitol and sufficient $CaCl_2$ to achieve pCa 4.5. Ca^{2+}-dependent Tyr and bis-1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS from Molecular Probes) fluorescence were performed as described previously [10,11]. Protein solutions for all fluorescence experiments contained 50 mM MOPS, pH 7.0, 150 mM KCl, 0.5 mM EGTA. Protein concentrations in all fluorescence experiments were 0.27 mg/ml. The final concentration of total $CaCl_2$ needed to achieve the desired free Ca^{2+} concentration in the myofibril and fluorescence assays was determined using the multiple equilibrium computer programs of Fabiato [12]. Apparent binding constants at pH 7.0 and 23°C for all metal ligands were calculated using these computer programs. The apparent binding constant for EGTA was 2.53×10^6. The concentration of contaminating Ca^{2+} in the buffers was about 5×10^{-6} M as determined using a Ca^{2+}-sensitive electrode (Orion) and Ca^{2+}/EGTA standards.

3. Results

3.1. N-Terminal mutants

The primary sequences of proteins with modifications in the N-terminus are shown in Fig. 1A. Expression plasmids for cTnCN1 and cTnCN3 were generated in a previous study [9]. In cTnCN1, the first 11 amino acids of native cTnC were replaced by the first 12 amino acids from helix A of vertebrate CaM. The primary sequence

*Corresponding author. Fax: (1) (713) 794-4150.

SSDI 0014-5793(94)00526-2

A. Comparison of N-terminal Amino Acid Sequences

	1
cTnC	M D D I Y K A A V E Q L T E
cTnC3	- A . . . . .
cTnCN1	A . Q L T E E Q I A E . . .
cTnCN3	A . Q . D . . . . .
cTnC(FLAG)	M D Y K D D D D K . . . . .
cTnC(ΔN)	- - - - - - - - - - A . . .
sTnC	. T . Q Q A E . R S Y . S .

B. Western Dot-blot for FLAG Epitope

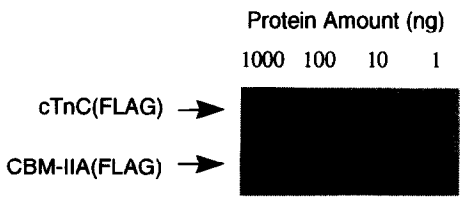

Fig. 1. Recombinant proteins. (A) The N-terminal amino acids of cTnC and sTnC are compared to the recombinant proteins used in this study. The numbering system is relative to tissue-derived cTnC which retains the N-terminal Met residue. The presence or absence of the N-terminal Met residue in the recombinant proteins was confirmed by amino acid sequencing or by antibody cross-reactivity for cTnC(FLAG). Dots indicate residues that are the same as in cTnC. Dashes indicate deleted residues. The sequence for sTnC is from the human and rabbit proteins [21]. (B) Purified proteins were diluted with 0.05 M Tris, pH 7.4, 0.15 M NaCl such that the indicated quantities of purified protein in a volume of 0.1 ml were applied to Nytran nitrocellulose membranes. The membranes were then allowed to dry and were exposed to the M2 FLAG antibody (IBI/Kodak) followed by an anti-rabbit antibody horseradish peroxidase antibody.

of the N-terminus of cTnCN3 most closely resembles normal recombinant cTnC3. cTnC(FLAG) and CBM-IIA(FLAG), which has an inactive site II, were generated to more rigorously test the requirement for a specific N-terminal primary sequence in cTnC and to provide a sensitive epitope tag on cTnC for other studies. In these constructs the first eight amino acids of the normal cTnC were replaced with the highly charged sequence MDYKDDDDK, which specifies the FLAG epitope (IBI/Kodak) and an enterokinase cleavage site. Fig. 1B shows that as little as 10 ng of purified cTnC proteins with the FLAG epitope can be detected by a commercially available M2 monoclonal antibody. The FLAG proteins do not cross-react with the M1 antibody (data not shown) which will only recognize the FLAG epitope if the initiator Met is absent.

Figure 2A shows the maximal levels of ATPase activity of TnC-extracted fast skeletal and cardiac muscle myofibrils that were reconstituted with cTnC3 or the various N-terminal mutants. cTnC(ΔN) was able to recover only about 40% of the activity recovered by cTnC3 in both cardiac and fast skeletal muscle myofibrils. All other mutants, even cTnC(FLAG), recovered levels of ATPase activity similar to, or slightly greater than, the activity recovered by cTnC3. The lowered activity of cTnC(ΔN) does not appear to be due to a less efficient incorporation of the protein into the myofibrils since concentrations of cTnC(ΔN) of up to 0.15 mg/ml did not increase the recovered activity (data not shown). Fig. 2B shows that all the N-terminal mutants, even cTnC(ΔN), exhibited similar Ca^{2+}-dependent activation characteristics. The relative pCa_{50} values appear to correlate with the relative severity of the mutations. cTnC3 and cTnCN3, which differ by only 4 amino acids, have identical pCa_{50} values of 5.7, while cTnC(ΔN) and cTnC(FLAG) have slightly lower pCa_{50} values of 5.5.

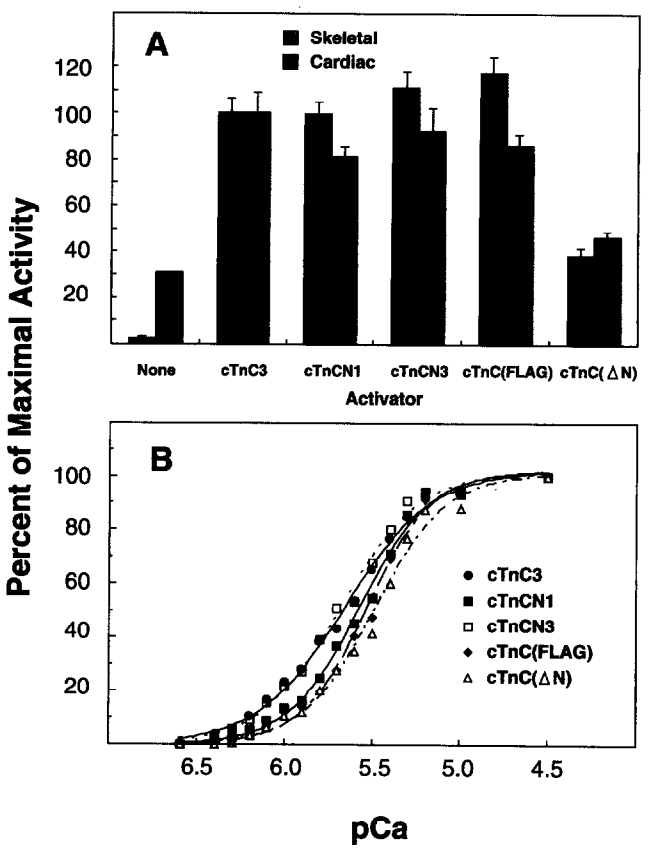

Fig. 2. Fast skeletal and cardiac myofibril ATPase activities of mutant proteins with N-terminal peptide modifications. (A) Myofibril extracts of endogenous TnC were reconstituted with no activator, cTnC3, cTnCN1, cTnCN3, cTnC(FLAG) or cTnC(ΔN). Ca^{2+}-dependent activity is defined as the difference in specific activity at pCa 3.6 and 8.0. Activity is expressed as the percent of the maximal Ca^{2+}-dependent activity observed for cTnC3 (about 100 nmol P_i/min/mg myofibril protein in skeletal myofibrils and 12 nmol P_i/min/mg myofibril protein in cardiac). (B). ATPase activity of myofibrils reconstituted with cTnC3, cTnCN1, cTnCN3, cTnC(FLAG) or cTnC(ΔN) was measured in the presence of increasing amounts of Ca^{2+}. The data are expressed as the percent of the maximal Ca^{2+}-dependent activity for each cTnC derivative. The data was fitted by least squares minimization to the following form of the Hill equation, $A/A_{max} = 1/(1 + 10^{n(\log Ca_{50} - \log Ca)})$ where A is Ca^{2+}-dependent activity at a given log Ca^{2+}, A_{max} is the maximal activity, n is the Hill coefficient and log Ca_{50} is the log of the Ca^{2+} concentration at which the activity is half maximal. Ca^{2+}-dependent activity is defined as the difference in specific activity at the indicated pCa and pCa 8.0.

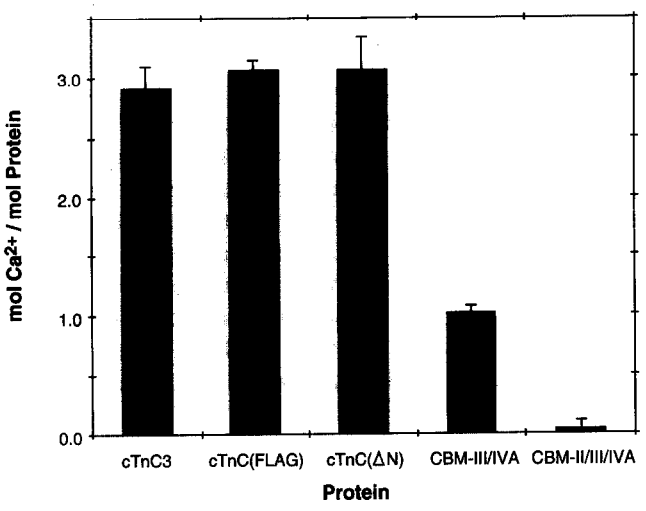

Fig. 3. Ca^{2+}-binding capacity of cTnC and its modified derivatives. Equilibrium dialysis was performed as described in section 2. $CaCl_2$ was added to achieve pCa 4.5. Protein concentrations were 1–2 mg/ml. The data represent the mean ± S.D. of duplicate determinations.

Based on the functional characteristics shown in Fig. 2, cTnC(⊿N) and cTnC(FLAG) were selected for biochemical characterization. Fig. 3 shows the Ca^{2+}-binding capacity of cTnC(⊿N) and cTnC(FLAG) determined by equilibrium dialysis at a free Ca^{2+} concentration of 3.16×10^{-5} M (pCa 4.5). Both proteins bound 3 mol Ca^{2+} per mol protein. The control proteins cTnC3, CBM-III/IVA and CBM-II/III/IVA bound 3, 1 and 0 mol Ca^{2+} per mol protein as predicted from previously studies [10,13].

cTnC3, cTnC(FLAG) and cTnC(⊿N) were titrated with Ca^{2+} while monitoring changes in intrinsic Tyr fluorescence or changes in fluorescence from the soluble probe bis-ANS which binds to hydrophobic surfaces on proteins. Fig. 4A, shows the effect of increasing concentrations of Ca^{2+} on fluorescence from bis-ANS in the presence of cTnC3, cTnC(FLAG) and cTnC(⊿N). The initial fluorescence, in arbitrary units, at pCa 8.5 was 370, 240 and 480 for cTnC3, cTnC(FLAG) and cTnC(⊿N), respectively. Titration of both cTnC3 and cTnC(FLAG) with Ca^{2+} caused bis-ANS fluorescence transitions that had pCa_{50} values of 7.0 and 5.3 (see Table 1), which correspond to the high and low affinity sites in cTnC [14]. Steady-state fluorescence continues to increase at pCa values greater than 4.0. This phenomenon, which has been observed previously with cTnC labeled with 2-(4′-iodoacetamido)anilino)naphthalene-6-sulfonic acid [9,15], may be due to non-specific binding of Ca^{2+} to clusters of acidic amino acids. Titration of cTnC(⊿N) with Ca^{2+} resulted in a bis-ANS fluorescence decrease that had a pCa_{50} of 7.09, and a subsequent gradual increase in fluorescence from pCa 6.0 to 3.0.

Similar to our previous results [10], we were unable to reproducibly detect a change in Tyr fluorescence from cTnC3 in response to Ca^{2+} binding to the low affinity site (see Fig. 4B). This was also true for cTnC(FLAG) which is missing Tyr^5 but has a Tyr residue in the FLAG epitope. Both the absolute increase in Tyr fluorescence and the pCa_{50} values were essentially identical for cTnC3 and cTnC(FLAG), and corresponded to Ca^{2+} binding to high affinity sites. Titration of cTnC(⊿N) with Ca^{2+} resulted in an increase in Tyr fluorescence with a pCa_{50} that was similar to that observed with bis-ANS and slightly greater than the pCa_{50} values for the high affinity sites in cTnC3 and cTnC(FLAG).

4. Discussion

In this study we have attempted to evaluate the functional significance of the N-terminal helix in cTnC. Our primary conclusion is that the presence but not the primary sequence of the N-terminal peptide in cTnC is essential for function. Even cTnC(FLAG) and cTnCN1, in which the first 12 amino acids differ greatly from those of cTnC3, show very little functional difference from cTnC3 in the myofibril ATPase assays. Only when the N-terminal peptide is deleted is there a sinificant decrease

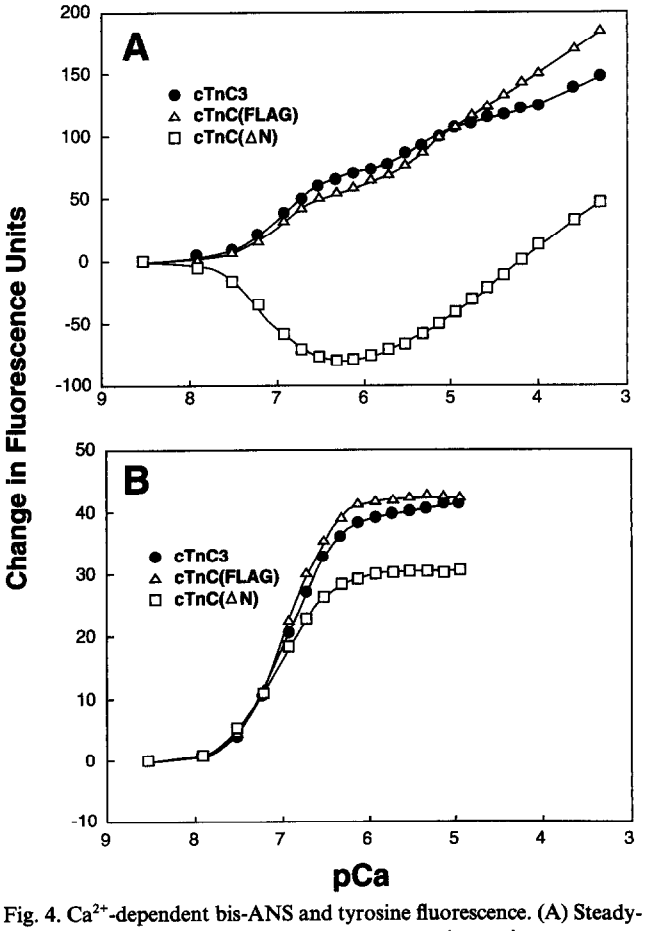

Fig. 4. Ca^{2+}-dependent bis-ANS and tyrosine fluorescence. (A) Steady-state fluorescence from bis-ANS fluorescence at increasing concentrations of free Ca^{2+} in the presence of the indicated proteins. (B) The effect of increasing free Ca^{2+} concentrations on the intrinsic Tyr fluorescence from the indicated proteins.

in maximal ATPase activity. This conclusion is contradictory to that reached by Gulati et al. [16]. The basis for these divergent conclusions may reside in differences in experimental design. For the current study, we chose to modify only the N-terminal peptide, within the context of normal cTnC and without modifications to other regions of the primary sequence. Gulati et al. chose to use chimeric cardiac/skeletal recombinants in which the primary sequence was mostly derived from sTnC. Specifically, the Ca^{2+} binding domain I of sTnC was replaced with the corresponding region of cTnC to generate a chimera with domain I of cTnC and domains II, III and IV from sTnC. The activity of this chimera was decreased substantially when it was further modified by replacing the first 10 amino acids with the corresponding amino acids from sTnC. The relevance of this data to the function of normal cTnC must be extrapolated cautiously since the data was obtained using a chimeric protein that derives less than 20% of its primary sequence from cTnC and in which normal interactions between Ca^{2+}-binding loops I and II, which are different in cTnC vs. sTnC [17,18], may not occur. It is possible that the observations of Gulati et al. will apply to sTnC but not cTnC.

Several explanations may account for the lowered activity of cTnC(ΔN). The simplest is that deletion of the N-terminus induces undesirable structural perturbations in cTnC. This possibility cannot be excluded without extensive biophysical characterization of cTnC(ΔN). However, if deletion of the N-terminal peptide caused improper protein folding, then it is likely that the Ca^{2+}-binding properties of the low affinity site II would be greatly affected. Deletion of the N-terminal helix altered the interaction of cTnC(ΔN) with bis-ANS such that no clear change in fluorescence was observed upon Ca^{2+} binding to the low affinity site. However, direct determination of Ca^{2+} binding by equilibrium dialysis showed that cTnC(ΔN) binds three mol Ca^{2+} per mol protein at pCa 4.5. Although we cannot exclude the possibility that deletion of the N-terminal helix causes inactivation of site II and activation of site I, it is more likely that sites II, III and IV in cTnC(ΔN) are saturated with Ca^{2+} at pCa 4.5. Moreover, the pCa_{50} for activation of myofibril ATPase by cTnC(ΔN) was identical to cTnC(FLAG) and only slightly different from cTnC3. If cTnC(ΔN) did not bind Ca^{2+} at site II, then it would be functionally inactive as we have shown previously for cTnC [9]. Thus, deletion of the N-terminal helix does not appear to destabilize the structure of site II to the extent that it has greatly different Ca^{2+}-binding properties.

A second possibility is that removal of the N-terminal peptide prevents specific interactions between this region and other regions in cTnC, or other thin filament proteins that are necessary for efficient transmission of the Ca^{2+} signal. This mechanism would be inconsistent with the maximal activities of cTnCN1 and cTnC(FLAG), since specific interactions would be expected to be disrupted by such severe mutations.

A third possibility is that the N-terminal peptide interacts relatively non-specifically with other regions in cTnC or other troponin subunits. Deletion of the N-terminus would prevent this and possibly allow other, functionally deleterious interactions to occur. Altered interactions within cTnC are suggested by Ca^{2+}-dependent changes in bis-ANS and Tyr fluorescence. Table 1 shows that cTnC3, cTnC(FLAG) and cTnC(ΔN) showed fluorescence transitions with pCa_{50} values that were consistent with the binding constants for high affinity sites III and IV in cTnC as determined by equilibrium dialysis [14]. The pCa_{50} for the high affinity sites in cTnC(ΔN) was slightly greater than the corresponding pCa_{50} values of cTnC3 and cTnC(FLAG). This increase in the apparent Ca^{2+}-binding affinity is consistent with the increased affinity of sites III and IV seen for recombinant C-terminal fragments of cTnC [10] and sTnC [19]. This suggests that there is an interaction between the N- and C-terminal domains in the free form of cTnC which involves, at least in part, the N-terminal peptide.

The effect of deletion of the N-terminal peptide on fluorescence from bis-ANS suggests that a hydrophobic surface is exposed in cTnC(ΔN). Analysis of a model for the predicted tertiary structure of cTnC [20] shows that deletion of the first 10 amino acids would cause a significant increase in the exposure of Val^{79} to solvent. In CaM, which does not have an N-terminal extension, there is a polar Thr residue at the position corresponding to Val^{79} in cTnC. A reasonable hypothesis is that exposure of the hydrophobic Val residue compromises the function of cTnC. To test this we engineered both cTnC and cTnC(ΔN) to covert Val^{79} to Thr. This modification resulted in only a slight increase in the Ca^{2+}-dependent activity of both cTnC3 and cTnC(ΔN) (data not shown). It is possible that exposure of other residues by themselves, or in synergy, cause the marked decrease in activity of cTnC(ΔN) relative to wild-type cTnC.

Acknowledgements: This work was supported in part by grants to J.A.P. from the National Institutes of Health (HL-45724) and the Welch Foundation. J.A.P. is the recipient of a Research Career Development Award from the National Institutes of Health.

References

[1] Collins, J.H., Greaser, M.L., Potter, J.D. and Horn, M.J. (1977) J. Biol. Chem. 252, 6356–6362.
[2] Van Eerd, J.P. and Takahashi, K. (1976) Biochemistry 15, 1171–1180.
[3] Dedman, J.R., Jackson, R.L., Schreiber, W.E. and Means, A.R. (1978) J. Biol. Chem. 253, 343–346.
[4] Herzberg, O. and James, M.N. (1985) Nature 313, 653–659.
[5] Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S.T., Roychowdhury, P., Greaser, M. and Wang, B.C. (1985) Science 227, 945–948.

[6] Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R. and Cook, W.J. (1985) Nature 315, 37–42.
[7] Morimoto, S. and Ohtsuki, I. (1988) J. Biochem. 104, 149–154.
[8] Babu, A., Orr, G. and Gulati, J. (1988) J. Biol. Chem. 263, 15485–15491.
[9] Putkey, J.A., Sweeney, H.L. and Campbell, S.T. (1989) J. Biol. Chem. 264, 12370–12378.
[10] Dotson, D.G. and Putkey, J.A. (1993) J. Biol. Chem. 268, 24067–24073.
[11] Putkey, J.A., Dotson, D.G. and Mouawad, P. (1993) J. Biol. Chem. 268, 6827–6830.
[12] Fabiato, A. (1988) Methods Enzymol. 157, 378–417.
[13] Negele, J.C., Dotson, D.G., Liu, W., Sweeney, H.L. and Putkey, J.A. (1992) J. Biol. Chem. 267, 825–831.
[14] Holroyde, M.J., Robertson, S.P., Johnson, J.D., Solaro, R.J. and Potter, J.D. (1980) J. Biol. Chem. 255, 11688–11693.
[15] Johnson, J.D., Collins, J.H., Robertson, S.P. and Potter, J.D. (1980) J. Biol. Chem. 255, 9635–9640.
[16] Gulati, J., Babu, A. and Su, H. (1992) J. Biol. Chem. 267, 25073–25077.
[17] Tsuda, S., Hasegawa, Y., Yoshida, M., Yagi, K. and Hikichi, K. (1988) Biochemistry 27, 4120–4126.
[18] Krudy, G.A., Brito, R.M.M., Putkey, J.A. and Rosevear, P.R. (1992) Biochemistry 31, 1595–1602.
[19] Li, M.X., Chandra, M., Pearlstone, J.R., Racher, K.I., Trigo-Gonzalez, G., Borgford, T., Kay, C.M. and Smillie, L.B. (1994) Biochemistry 33, 917–925.
[20] Brito, R.M.M., Putkey, J.A., Strynadka, N.C.J., James, M.N.G. and Rosevear, P.R. (1991) Biochemistry 30, 10236–10245.
[21] Collins, J.H. (1991) J. Muscle Res. Cell Motil. 12, 3–25.