A Structural Role for the Ca\(^{2+}\)-Mg\(^{2+}\) Sites on Troponin C in the Regulation of Muscle Contraction

PREPARATION AND PROPERTIES OF TROPONIN C DEPLETED MYOFIBRILS

Henry G. Zot and James D. Potter‡

From the Section of Contractile Proteins, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Troponin C (TnC)-depleted myofibrils from rabbit skeletal muscle were prepared by consecutive washes of whole myofibrils with EDTA in solutions of low ionic strength, a method which has recently been described for the preparation of TnC (Cox, J. A., Compte, M., and Stein, E. A. (1981) Biochem. J. 195, 205-211). Myofibrils treated in this manner exhibit a calcium independent loss of myofibrillar ATPase activity which is fully reversible with addition of TnC. Extractions of whole myofibrils, with metal chelators under conditions in which the Ca\(^{2+}\)-specific or Ca\(^{2+}\)-Mg\(^{2+}\) sites of TnC have different metal occupancies, demonstrate that the removal of metal ions from the Ca\(^{2+}\)-Mg\(^{2+}\) sites is responsible for the dissociation of TnC from myofibrils and that binding of either Ca\(^{2+}\) or Mg\(^{2+}\) to the Ca\(^{2+}\)-Mg\(^{2+}\) sites prevents this dissociation. The extent of TnC dissociation from myofibrils with EDTA can be modulated by raising the KCl concentration. At higher KCl concentrations, a smaller percentage of TnC is extracted by EDTA. The TnC interaction with the Tn complex within myofilaments must be thus mediated by at least two mechanisms. One type of interaction is best demonstrated by a KCl modulated interaction in the absence of divalent metal ions. Another type of interaction, which can be best detected at lower KCl concentrations, involves Ca\(^{2+}\) and/or Mg\(^{2+}\) binding to the Ca\(^{2+}\)-Mg\(^{2+}\) sites. Although both of these mechanisms probably function in the intact muscle cell, the effect of Ca\(^{2+}\) and Mg\(^{2+}\) on the TnC and TnI interaction is most important since it is this interaction which is thought to be one of the primary steps in the regulatory event of the contraction cycle. Since previous studies (Robertson, S. P., Johnson, J. D., and Potter, J. D. (1981) Biophys. J. 34, 559-589) have shown that the Ca\(^{2+}\)-Mg\(^{2+}\) sites always contain either Ca\(^{2+}\) or Mg\(^{2+}\) in vivo, we conclude that the Ca\(^{2+}\)-Mg\(^{2+}\) sites of TnC play a structural role in maintaining the integrity of the troponin molecule.

Troponin, a protein comprised of three nonidentical subunits (1), is the primary calcium-sensitive regulatory compo-

* Supported by American Heart Association Grant 78-1167 and National Institutes of Health Grant HL 26193-3A. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed at the Section of Contractile Proteins, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267.

(Received for publication, November 23, 1981, and in revised form, March 8, 1982)
TnT (13, 14), TnC and TnI (15), and the TnC-TnI binary complex and actin (7) all are mediated by Ca$^{2+}$ binding to TnC. TnC is known to bind 4 mol of Ca$^{2+}$/mol of TnC, 2 mol to each of 2 classes of sites (4). The first class of sites binds Ca$^{2+}$ with high affinity and also binds Mg$^{2+}$ competitively. These high affinity sites are referred to as Ca$^{2+}$-Mg$^{2+}$ sites (4, 16). The other class of sites on TnC are named Ca$^{2+}$ specific sites since they are distinguished from the Ca$^{2+}$-Mg$^{2+}$ sites, having a lower affinity for Ca$^{2+}$ and not binding Mg$^{2+}$ under physiological conditions (4). Since a large body of evidence (reviewed by Potter and Johnson (17)) supports the Ca$^{2+}$-specific sites alone as the regulatory sites for muscle contraction (4), this Ca$^{2+}$ signal could be transmitted by Ca$^{2+}$- dependent interactions with other Tn subunits. Having apparently no regulatory function in muscle contraction despite inducing large changes in conformation upon binding Ca$^{2+}$ or Mg$^{2+}$ (18, 19), the Ca$^{2+}$-Mg$^{2+}$ sites have been assigned a structural function (4, 20, 25). Supporting evidence for this view is based on the observation that divalent metal ions, presumably by binding to the Ca$^{2+}$-Mg$^{2+}$ sites of TnC, exert a stabilizing effect on the Tn complex (specifically the TnI-TnC binary complex) such that EDTA is necessary to completely separate TnI, TnC, and TnC in 6 M urea (12, 15) and the Ca$^{2+}$-Mg$^{2+}$ sites are probably occupied by divalent metal ions throughout the contraction cycle (26). However, no direct evidence has been accumulated to substantiate this role for the Ca$^{2+}$-Mg$^{2+}$ sites of TnC.

METHODS

Preparation of Myofibrils—Myofibrils were prepared from rabbit fast back muscle as closely as possible to the method described by Cox et al. (10). After the preliminary washes, the myofibrils were extracted 7 times with 2 volumes (based on the starting weight of muscle) of extraction buffer containing 20 mM Tris HCl, pH 7.8, 15 mM 2-mercaptoethanol, 20 mM PMSEF, 100 μg/liter of pepstatin A, and 0.6 mM NaN$_3$. Additions of chelator and cation to this basic solution were made as described in the figure legends. Extractions were performed at 0–4 °C by homogenization, followed immediately by centrifugation at 2000 × g for 5 min. After the extraction (with or without chelator), the myofibrils (both control and chelator-treated) were homogenized and centrifuged as above 4 times with 4 volumes of 10 mM MOPS, pH 7.0, 90 mM KCl, 0.1% NaN$_3$, and 0.1 mM dithiothreitol. Experiments were carried out within 6 days of myofibril preparation since at this time the myofibrils were stored at 0–4 °C without chelator.

ATPase Assay—2-ml reaction mixtures were prepared by adding 1 mg of myofibrils to 120 mM MOPS, pH 7.0, 90 mM KCl, 2 mM EGTA, 5 mM MgCl$_2$, and 0.1 mM dithiothreitol. Calcium was added to each reaction tube in order to achieve the desired free calcium concentration. The reaction was initiated by addition of ATP (to 2 mM) and was run at ambient temperature until terminated with ice-cold 6% trichloroacetic acid. ATPase activity was determined from the inorganic phosphate (P$_i$) released (27). Activities were plotted as per cent maximum activity which is equal to A/A' × 100 where A is the ATPase activity at a given pCa and A' is the maximum ATPase activity. For each experiment, the specific activity (μmol of P$_i$/mg/min) for A' is included in the figure legend.

SDS-PAGE—The protein content of the supernatants, which contained the extracted proteins, and the pellets, which contained the treated myofibrils, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 20-cm glass tubes (28). The supernatants from the extraction were combined and kept on ice no more than 5 h before being centrifuged at 55,000 × g for 15 h at 4 °C. The purpose of this step was to remove any small myofibrillar fragments or thin filaments present in the supernatants. The protein in the supernatant from the high speed centrifugation was either precipitated by making the solution 6% trichloroacetic acid and incubating overnight at 4 °C or concentrated by ultrafiltration using an Amicon ultrafiltration apparatus with a PM 10 filter. Identical gel profiles were produced. Gel samples of the extract or the treated myofibrils were prepared by dialyzing the buffer or trichloroacetic acid away from the protein prior to adjusting the pH for electrophoresis.

Densitometry—SDS gels were scanned for absorbance with a Soft Laser Scanning Densitometer (Biomed Instruments) using the tungsten lamp and a 520-nm filter. Selected gels from each electrophoresis run were scanned as a group. The photomultiplier gain was empirically set to optimum; the signal gain was adjusted so that peaks in the area of the gel of interest were on scale, and the zero was adjusted to a blank area on the gel. All adjustments were performed on the first gel of the set of gels to be scanned and these settings locked in place throughout the group of gels scanned. Since the gel profiles were linearly ordered on the same strip of chart paper, comparative scans were traced onto the same sheet of paper from the original using the same chart paper scales as templates.

RESULTS

In order to determine the effect of TnC removal on myofibrillar ATPase, myofibrils were treated, as described under “Methods,” either in the presence of EDTA (depleted) or in the absence of a chelator (control). Measurement of ATPase activity (Fig. 1) of the resulting myofibrils demonstrated a substantial loss in activity for depleted myofibrils at nearly all free calcium levels compared to the control, suggesting that TnI was inhibiting activation. Inspection of SDS gels of the extracted protein revealed a large band corresponding to TnC in the supernatant from the depleted myofibrils (Fig. 2, gel 2), which was only faintly present in the extract of the control (Fig. 2, gel 1). A faint band corresponding probably to LC$_2$ or calmodulin was found to be extracted with EDTA (Fig. 2, gel 3) which was absent in the soluble extract from control myofibrils (Fig. 2, gel 1). This small amount of protein extracted is probably insufficient to cause the large changes in Mg$^{2+}$ ATPase observed. Thus, although several proteins were extracted by treatment of the myofibrils with EDTA, only TnC appeared to be extracted in relatively sufficient quantities to cause the depression in the activation.

Concurrently, the amount of TnC relative to TnI in myofibrils treated with EDTA was compared to the relative amount of TnC in control myofibrils. In Fig. 3, a representative den-
The Role of the Ca$^{2+}$ and Mg$^{2+}$ Sites on Troponin

calcium regulation by reconstituting depleted myofibrils with purified TnC. At pCa 4.0 (Fig. 4), the level of ATPase of depleted myofibrils was fully restored to the same level as control myofibrils with increasing additions of TnC. Furthermore, there was no effect of TnC on the control myofibrillar ATPase, eliminating the possibility that free TnC by itself stimulated the ATPase. The reconstitution of the activation of myofibrillar ATPase by pure TnC was fully retained after washing of the reconstituted myofibrils in the presence of metal ions (data not shown). These results demonstrate that TnC alone is responsible for the loss in activation and that, since the loss was reversible with the addition of pure TnC, the depleted myofibrils were probably structurally intact.

Extraction of TnC with EDTA suggests that the metal-binding sites of TnC are involved in the binding of TnC to the TnT-TnI binary complex. Using metal chelators, i.e. EDTA and EGTA, the free Ca$^{2+}$ and Mg$^{2+}$ present during extractions were adjusted so that the Ca$^{2+}$-specific or Ca$^{2+}$-Mg$^{2+}$ sites of TnC would be occupied to different degrees (Table I). Myofibrils treated in this manner were assayed for calcium activa-

**Fig. 2. SDS-PAGE of the EDTA-treated and control supernatants and myofibrils from Fig. 1.** Samples were prepared as described under "Methods." 1, control supernatant; 2, control myofibrils; 3, EDTA-treated supernatant; 4, EDTA-treated myofibrils; 5, total myosin light chain fraction; 6, purified TnC standard; 7, purified calmodulin plus TnC standard.

Densitometric scan of the myofibrils is shown. Although there is a significant band corresponding to TnC shown on the gel from TnC-depleted myofibrils, it is consistently reduced compared to gels of control myofibrils. This qualitative assessment of the difference in TnC content observed on SDS gels compared to the apparently larger quantitative difference in myofibrillar ATPase activities at high Ca$^{2+}$ (demonstrated in Fig. 1 for the control and TnC depleted myofibrils) might suggest that TnC may not be related to the loss in activity. However, it is well known that Coomassie blue binding to proteins is difficult to quantitate and the staining of TnC is reduced compared to other myofibrillar proteins (32). It is possible that the small differences in the relative intensity of TnC bands reflect a nonlinear binding of stain rather than the actual difference in TnC content. Due to these problems in examining the myofibrils, the best way to judge the loss of TnC is to examine the gels of the concentrated extracts which unequivocally demonstrate the extraction of TnC from myofibrils.

If TnC were indeed responsible for the loss of Ca$^{2+}$ sensitivity seen in Fig. 1, then it should have been possible to regain

**Fig. 3. A densitometric scan of SDS gels of the EDTA-treated and control myofibrils.** Aliquots of EDTA-treated and control myofibrils were taken from the preparations used in Fig. 1 and prepared for electrophoresis as described in the text. The scan shows the densities of the resultant stained protein bands in the region of the gel which contained the myosin light chains (LC: at relative migration 6.0, LC$_1$ at 4.0, and LC$_2$ at 2.5) and TnC and TnI (at relative migrations 4.5 and 5.7, respectively). ---, EDTA-treated; - - -, control; ↓, location of pin; ↓, location of TnC; *, location of TnI.

**Fig. 4. The reconstitution of EDTA-treated myofibrils with TnC.** Pure TnC was added directly to the ATPase assay reaction mixture containing either TnC-depleted or control myofibrils and Mg$^{2+}$ under the assay conditions described under "Methods." TnC was added to both the EDTA-treated (●) and control (○) myofibrils at both pCa 4.0 (——) and pCa 7.0 (---). A' was 0.250 μmol P/mg/min.
tors (EGTA and EDTA), when used, were present.

The concentration of the solutions used for these extractions. The chelator (EGTA and EDTA), when used, were present as 5 mM. The extraction conditions were the same as described under “Methods.” The number of the extraction solution corresponds with the number in Table I for the free metal concentration of the solutions used for these extractions. The chelators (EGTA and EDTA), when used, were present as 5 mM. The extraction conditions were the same as described under “Methods.” The number of the extraction solution corresponds with the number in Table I for the following: 1, control; 2, 4, 6, 2, and 3, 3. A was 0.211 μmol P/mg/min.

The effect of Ca2+ and Mg2+ concentration on the extraction of TnC from myofibrils. See Table I for the free metal concentration of the solutions used for these extractions. The chelators (EGTA and EDTA), when used, were present as 5 mM. The extraction conditions were the same as described under “Methods.” The number of the extraction solution corresponds with the number in Table I for the following: 1, control; 2, 4, 6, 2, and 3, 3. A was 0.211 μmol P/mg/min.

When Ca2+ was present in both the Ca2+-specific and Ca2+-Mg2+ sites, the removal of TnC from the myofibrils was prevented during the time of these extractions. Reconstitution to the control level ATPase with pure TnC (Table II) shows that some TnC is removed at all KCl concentrations tested. SDS gels of trichloroacetic acid precipitable material in the extracted volume showed that TnC was removed in each case in proportion to the activity lost when compared with the control gel (data not shown).

Thus, unlike the strong affinity of TnC for the TnT-TnI binary complex when the metal binding sites of TnC are occupied, increasing the ionic strength alone acts to stabilize a weaker interaction.
The dissociation of TnC from myofibrils demonstrates a highly specific process for the action of EDTA. All of the supernatants show essentially the same relative amounts of proteins other than TnC, regardless of how the myofibrils were treated (e.g. control, EDTA, etc.). TnC, however, is the only protein which is extracted with treatment by EDTA or EGTA and, thus, is specifically extracted. As the gels of the soluble extract in Fig. 2 (gels 1 and 3) illustrate, compared with the control myofibrils, only TnC appears to be extracted with EDTA. Further evidence that the action of EDTA is highly specific comes from the reconstitution of TnC-depleted myofibrils with pure TnC. Assuming that activation is reconstituted in TnC-depleted myofibrils as described in Fig. 4.

**Table II**

<table>
<thead>
<tr>
<th>(KCl)$^a$</th>
<th>Depleted$^b$</th>
<th>Reconstituted$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.4$^c$</td>
<td>88.1</td>
</tr>
<tr>
<td>25</td>
<td>49.6</td>
<td>107.2</td>
</tr>
<tr>
<td>50</td>
<td>72.0</td>
<td>106.4</td>
</tr>
<tr>
<td>100</td>
<td>81.8</td>
<td>91.3</td>
</tr>
<tr>
<td>150</td>
<td>82.9</td>
<td>98.4</td>
</tr>
</tbody>
</table>

$^a$ KCl concentration used in the extraction solution.

$^b$ Depleted myofibrils prepared as described under "Methods" with 5 mM EDTA and varying amounts of KCl present during extraction.

$^c$ All reconstitutions were performed by adding 0.5 μmol of pure TnC/g of myofibrils as described in Fig. 4.

Control myofibrils prepared in the same manner as described for control myofibrils in Fig. 1. $A' = 0.250 \mu mol$ P$_{i}$/mg/min.

Addition of the same concentration of TnC was made to control myofibrils as that added to depleted myofibrils. $A' = 0.290 \mu mol$ P$_{i}$/mg/min.

$^d$ ATPase activity measured as described for Fig. 1 at pCa 5.0.

**DISCUSSION**

The dissociation of TnC from myofibrils demonstrates a highly specific process for the action of EDTA. All of the supernatants show essentially the same relative amounts of proteins other than TnC, regardless of how the myofibrils were treated (e.g. control, EDTA, etc.). TnC, however, is the only protein which is extracted with treatment by EDTA or EGTA and, thus, is specifically extracted. As the gels of the soluble extract in Fig. 2 (gels 1 and 3) illustrate, compared with the control myofibrils, only TnC appears to be extracted with EDTA. Further evidence that the action of EDTA is highly specific comes from the reconstitution of TnC-depleted myofibrils with pure TnC. Assuming that activation is reconstituted in TnC-depleted myofibrils as described in Fig. 4, control myofibrils prepared in the same manner as described for control myofibrils in Fig. 1. $A' = 0.250 \mu mol$ P$_{i}$/mg/min.

Addition of the same concentration of TnC was made to control myofibrils as that added to depleted myofibrils. $A' = 0.290 \mu mol$ P$_{i}$/mg/min.

Based on the actin content of myofibrils and the molar ratio of actin and Tn, the Tn content of myofibrils is estimated to be 0.5 μmol/g (34). Assuming a 1:1 molar ratio of TnC to tropomyosin (32), the TnC content of myofibrils is also estimated to be 0.5 μmol/g. At pCa 4.0 (Fig. 4), depleted myofibrils were originally about 30% maximal activity which implies that 70% of the TnC was removed during extraction. If 70% of the estimated 0.5 μmol of the TnC/g of myofibrils is taken as the total TnC removed, then about 0.4 μmol of TnC/g of myofibrils would be necessary to restore full activity. This estimate of the amount of TnC removed during extraction is very close to the actual amount of TnC which restored full activity (Fig. 4). Therefore, the level of nonspecific interactions which occur upon TnC addition to depleted myofibrils is concluded to be small while the percentage of specificity that TnC has for Tn reconstitution approaches 100.

TnC-depleted myofibrils have characteristics which distinguish them from desensitized actomyosin. Extraction of myofibrils with 20 mM Tris HCl, pH 7.8, is shown here not to have an effect on the Ca$^{2+}$ regulation of activation (Fig. 1, control). Under similar conditions Schaub et al. (35) prepared desensitized actomyosin from natural actomyosin. The fraction released during low ionic strength wash of the natural actomyosin was subsequently found to contain the subunits of Tn (36, 37). Since intact myofibrils treated at low ionic strengths are shown here to have extracted virtually no Tn components (Fig. 2, gel 1), these myofibrils are probably biochemically distinct from natural actomyosin.

Reversible binding of TnC to the Ca$^{2+}$ regulatory site within native myofibrils has structural implications concerning the Tn subunit organization (Fig. 7). A characteristic of TnC-depleted myofibrils is the inhibition of activation in the presence of Ca$^{2+}$. Identical results were reported (36, 37) for desensitized actomyosin reconstituted with a fraction containing a complex of TnT and TnI, i.e. a Ca$^{2+}$ insensitive relaxation. The reassociation of TnC to TnC-depleted myofibrils must be through a metal ion dependent interaction with the TnT-TnI binary complex. We cannot readily distinguish to which subunit TnC actually binds, since Ca$^{2+}$-dependent interactions have been demonstrated to occur between TnT and TnC (13, 14) as well as TnI and TnC (15, 38). However, work by Ebashi (2) and our own unpublished observations suggest that cardiac TnC interacts only weakly with cardiac TnT. Furthermore, nonphysiological conditions are required to quantify the interaction between TnC and TnT (39). A non-specific interaction between TnC and TnT is supported by a recent cross-linking study (40) which suggests that while TnC and TnT are in close proximity in isolated Tn, upon reconstitution into thin filaments, TnC and TnT occupy a different orientation with respect to each other. Thus, we believe that TnC interacts directly with TnI under conditions where the Ca$^{2+}$-Mg$^{2+}$ sites are occupied and this interaction is fundamental to regulation, whereas that between TnC and TnT, if it occurs at all in the whole Tn complex, may not be important (Fig. 7).

Calcium binding to TnC and its effect on the interaction of TnC and TnI are of particular interest because structural changes in TnC modify not only the binding of TnI to actin, but also the interaction of TnC with TnI itself. Measurements of structural changes in TnC show that divalent metal binding to the Ca$^{2+}$-Mg$^{2+}$ sites cause large changes in secondary structure in TnC (23, 41) while Ca$^{2+}$ binding to the Ca$^{2+}$-specific sites probably affects the tertiary folding of the molecule (24). Conformational changes in TnC with Ca$^{2+}$ have been shown to be necessary for the interaction between TnC and TnI to take place in 6 M urea (15). Experiments with the region III fragment of TnC, i.e. CB9, which contains a Ca$^{2+}$-Mg$^{2+}$ site (42), show that this region binds TnI in a calcium-dependent manner (38). Further results demonstrate that region II, which contains a Ca$^{2+}$-specific site, also binds to TnI (43) leading to
The Role of the Ca$^{2+}$ and Mg$^{2+}$ Sites on Troponin

The dissociation of TnC is not likely to occur in the intact cell for at least two reasons. First, the intracellular ionic strength is high enough to prevent most of the loss of TnC from the myofilaments in the absence of divalent metal ions, possibly through the neutralization of charges within the Tn complex with subsequent alterations in the structure of one or more of the Tn subunits which might, for example, enhance hydrophobic interactions. Secondly, under physiological conditions, the Ca$^{2+}$-Mg$^{2+}$ sites would always be filled, either with Mg$^{2+}$ or Ca$^{2+}$ during the contraction-relaxation cycle (26). It is probably this second type of binding between TnC and TnI that Castellani et al. (44) found to distinguish calmodulin binding to TnI from that of TnC. In their hands, TnC bound independently of Ca$^{2+}$ (in the presence of Mg$^{2+}$), whereas calmodulin only bound in the presence of Ca$^{2+}$. Since calmodulin only has Ca$^{2+}$-specific sites and does not contain any Ca$^{2+}$-Mg$^{2+}$ sites (45), one would not expect it to bind in the absence of Ca$^{2+}$ even in the presence of Mg$^{2+}$. Thus, the earlier proposal (4, 20–25), that the primary role of the Ca$^{2+}$-Mg$^{2+}$ sites is to maintain a certain configurational state of 'TnC independent of the cytosolic levels of Ca$^{2+}$, is correct and results in the maintenance of the integrity of the troponin complex, allowing the Ca$^{2+}$-specific sites to function in the regulation of Ca$^{2+}$ activation. This technology also makes it possible to carry out a large number of experiment with one type of TnC present in either myofibrils, or even skinned fibers, can be altered and we are currently pursuing these lines of research.

REFERENCES

A structural role for the Ca2+-Mg2+ sites on troponin C in the regulation of muscle contraction. Preparation and properties of troponin C depleted myofibrils.
H G Zot and J D Potter


Access the most updated version of this article at http://www.jbc.org/content/257/13/7678.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/13/7678.citation.full.html#ref-list-1