A Disulfide Crosslink Between Cys^{98} of Troponin-C and Cys^{133} of Troponin-I Abolishes the Activity of Rabbit Skeletal Troponin

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ABSTRACT Various thio-reactive bifunctional crosslinkers as well as 5,5′-dithiobis(2-nitrobenzoate)-mediated disulfide bond formation were used to crosslink troponin-C and troponin-I, the Ca^{2+}-binding and inhibitory subunits of troponin, respectively. In all cases, substantial crosslinking was obtained when the reactions were carried out in the absence of Ca^{2+}. No disulfide crosslinking occurred if either Cys^{98} of TnC, or Cys^{133} of TnI were blocked, indicating that these thiols are involved in the crosslinking. Troponin containing the disulfide crosslink is no longer capable of regulating actomyosin ATPase activity in a Ca^{2+}-dependent manner. Our results suggest that the relative movement between the Cys^{98} region of TnC and the Cys^{133} region of TnI is required for the Ca^{2+}-regulatory process in skeletal muscle.

INTRODUCTION

Troponin (Tn) is the Ca^{2+}-binding thin filament protein complex that, in concert with tropomyosin (Tm), mediates regulation of vertebrate striated muscle contraction by Ca^{2+} (Ebashi and Endo, 1968). Tn is composed of three subunits, viz. TnC, TnI, and TnT, the Ca^{2+}-binding, inhibitory, and Tm-binding subunits, respectively. X-ray crystallography revealed that TnC is composed of an N- and a C-terminal domain well separated by a single central helix (Herzberg and James, 1985; Sundaralingam et al., 1985). The N-terminal domain contains the two low affinity Ca^{2+}-specific sites (designated as sites I and II), whereas the C-terminal domain contains the two high affinity Ca^{2+}/Mg^{2+} sites (designated as sites III and IV). There are four Cys's in Tn; Cys^{98} in TnC, Cys^{48}, Cys^{64}, and Cys^{133} in TnI; and none in TnT. It is currently thought that binding of Ca^{2+} to the low affinity triggering sites of TnC induces successive changes in the mode of interaction between TnC and TnI, TnI and actin, Tm and actin, and finally, myosin head and actin (reviewed by El-Saleh et al., 1986). In view of its crucial role in Ca^{2+} regulation, the interaction between TnC and TnI has been studied by many workers (reviewed by Leavis and Gergely, 1984; Zot and Potter, 1987; Grabarek et al., 1992). In particular, the region of TnC containing Cys^{98} has been shown to interact with the inhibitory region of TnI by studies employing proteolytic fragments (Grabarek et al., 1981), NMR (Dalgarno et al., 1982), and photocrosslinking (Leszyk et al., 1987, 1988). Furthermore, crosslinking between Cys^{98} of TnC and Cys^{133} of TnI by FNB was reported by Dobrovol'sky et al. (1984), whereas resonance energy transfer studies showed that the distance between these two residues decreases by ~5 Å in response to the binding of Ca^{2+} to TnC (Tao et al., 1989; Wang and Cheung, 1984).

In this study, we used a variety of crosslinkers to characterize further the proximity relationship between Cys^{98} of TnC and Cys^{133} of TnI in the ternary Tn complex. We found that the two residues can be crosslinked by sulfhydryl-specific bifunctional reagents with linker regions of various lengths and rigidity. Furthermore, a disulfide bond between them can be formed rapidly when Nbs_2 is used as a catalyst. The ternary Tn complex containing this disulfide bond is no longer capable of regulating actomyosin ATPase activity in a Ca^{2+}-dependent manner. The significance of these results with respect to the mechanism of action of Tn will be discussed.

Received for publication 20 December 1993 and in final form 17 March 1994.

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Abbreviations used: Tn, Tm, and S1: rabbit skeletal troponin, tropomyosin, and myosin subfragment 1, respectively. TnC, TnI, and TnT: the Ca^{2+}-binding, inhibitory, and Tm-binding subunits of Tn, respectively; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; Nbs_2, 5,5′-dithio-bis-2-nitrobenzoic acid; TNB^-, thionitrobenzoic acid ion; *p*-PDM, *N,N′-p*-phenyldimaleimide; *o*-PDM, *N,N′-o*-phenyldimaleimide; BMME, bis-(maleimide)-methyl ether; DMSDS, 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid; FNB, 1,3-difluoro-4,6-dinitrobenzene.

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0006-3495/94/06/2062/04 $2.00

MATERIALS AND METHODS

Chemicals

DMSDS was purchased from Molecular Probes (Junction City, OR). BMME was from Boehringer Mannheim (Indianapolis, IN). HEPES was from Research Organics (Cleveland, OH). Materials for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Protein preparation

Tn, Tn subunits, and Tm were prepared from rabbit skeletal ether powder as described in Greaser and Gergely (1973). Actin was extracted from rabbit

skeletal muscle acetone powder according to Spudich and Watt (1971). Myosin was prepared according to Balint et al. (1975), and chymotryptic S1 was prepared from myosin by the method of Weeds and Pope (1977).

Tn reconstitution

Tn was routinely reconstituted from the individual components shortly before crosslinking experiments. Equimolar amounts of TnC, TnI, and TnT were mixed in 6 M urea, 10 mM HEPES, pH 7.0 containing 0.1 M NaCl, 5 mM EDTA, and 5 mM DTT. After incubation for 1 h at room temperature, the proteins were slowly renatured and reconstituted by dialysis against 10 mM HEPES, pH 7.0 containing 0.1 M NaCl, 0.1 mM $CaCl_2$, and 1 mM DTT, followed by further dialysis against the same buffer, but without DTT.

Crosslinking by bismaleimides

Molar excess 1.2-fold of bismaleimide derivatives were added to the reduced and reconstituted Tn in 10 mM HEPES, pH 7.0, 0.1 M NaCl in the presence or absence of Ca^{2+}. After 30 min of incubation, the reaction was stopped by adding excess DTT, and the reaction mixture was analyzed by SDS-PAGE.

Sulfhydryl blocking

Cys^{98} of TnC was alkylated by adding iodoacetamide to denatured TnC at a molar ratio of 3:1 (in 4 M guanidinium chloride, 5 mM EDTA, 10 mM HEPES, pH 7.0) followed by incubation for 1 hour at room temperature. The reaction was stopped by the addition of excess DTT, and the unreacted reagents were removed by dialysis. Alkylation of Cys^{133} in TnI exploits the finding that only this TnI thiol is reactive in the ternary Tn complex (Chong and Hodges, 1982). Following a procedure that has been shown by Strasburg et al. (1985) to modify only Cys^{133} of TnI, reconstituted Tn was incubated with *N*-ethylmaleimide at a molar ratio of 5:1 and allowed to react for 4 h at room temperature. The reaction was quenched with excess DTT, and unreacted reagents were removed by dialysis.

Miscellaneous

Mg^{2+}-ATPase activity of regulated actomyosin was measured according to White (1982). SDS-PAGE was performed by the method of Laemmli (1970).

RESULTS

When reconstituted Tn was treated with a stoichiometric amount of Nbs_2 in the presence of Ca^{2+}, the absorption at 412 nm (A_{412}) rose rapidly, indicating the release of TNB^- (Fig. 1). The increase in A_{412} leveled off when approximately 1 mol of TNB^- per mol of Tn was liberated. SDS-PAGE of this reaction mixture revealed the formation of only a small amount of high molecular weight-crosslinked material (Fig. 2 *A*, lane 2). Addition of EGTA at this stage resulted in another rapid rise in A_{412}, indicating the release of a second mol of TNB^-. SDS-PAGE of the final reaction mixture revealed a high molecular weight band whose mobility is consistent with the molecular mass of the TnC-TnI complex (Fig. 2*A*, lane 3). Addition of DTT to this reaction mixture before SDS-PAGE completely removed the high molecular weight band (Fig. 2 *A*, lane 4), indicating that the complex was formed via a disulfide bond. When the high molecular weight band was excised from the gel, treated with DTT, and rerun on SDS-PAGE under reducing conditions, bands corresponding to TnC and TnI were observed (Fig. 2*B*, lane 3), confirming the notion that the crosslink is between TnC and TnI. If either Cys^{98} of TnC or Cys^{133} of TnI was pre-blocked with alkylating reagents, no crosslinking was observed (Fig. 2 *C*), indicating that the disulfide crosslink is between these two Cys's.

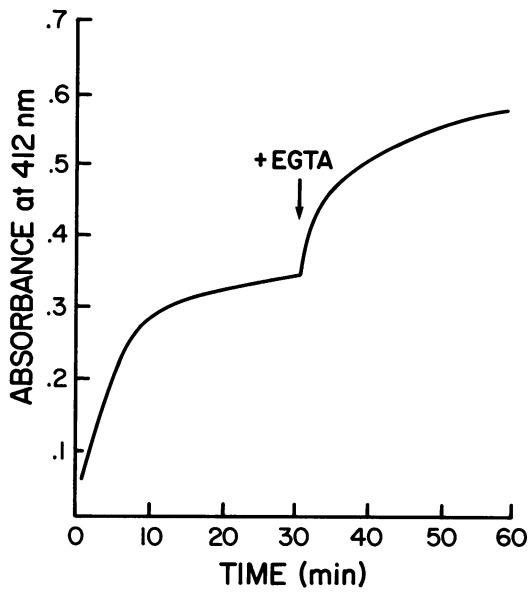

FIGURE 1 Time course of Nbs_2-catalyzed disulfide crosslinking between TnC and TnI monitored by the absorbance at 412 nm (A_{412}). An equimolar amount of Nbs_2 was added to freshly reconstituted Tn (20 μM) in a buffer containing 0.1 M NaCl, 0.1 mM $CaCl_2$, 10 mM HEPES, pH 7.0, at room temperature. At the time indicated by the arrow, 2 mM EGTA was added. Calculated A_{412} value for one mol of TNB^- ion released per mol of Tn is 0.272, using ϵ_{412} = 13,600 cm^{-1} M^{-1}.

The activity of the Tn containing this disulfide bond was assayed by measuring its ability to mediate Ca^{2+} regulation of acto-S1 ATPase in an in vitro system. As shown in Table 1, with increasing molar ratio of Nbs_2 to Tn the Ca^{2+}-regulatory capacity of the system decreased, indicating that formation of the TnC-TnI disulfide bond rendered the modified Tn incapable of inhibiting acto-S1 ATPase in the absence of Ca^{2+}.

A variety of bifunctional thio-reactive reagents were also used to crosslink TnC and TnI; these include FNB, *p*-PDM, *o*-PDM, BMME, and DMSDS. It can be seen that in the absence of Ca^{2+}, all these reagents are capable of mediating the crosslinking of TnC and TnI (Fig. 3).

DISCUSSION

It has been shown that of the four sulfhydryl groups in Tn, Cys^{48}, and Cys^{64} of TnI are buried and are unreactive regardless of Ca^{2+}, Cys^{133} of TnI is exposed and is reactive regardless of Ca^{2+}, and Cys^{98} of TnC is reactive only in the absence of Ca^{2+} (Chong and Hodges, 1982; Potter et al., 1976). Based on this information, we can reasonably propose the following scheme for our disulfide crosslinking reaction: 1) Incubation of Tn with Nbs_2 first in the presence of Ca^{2+}

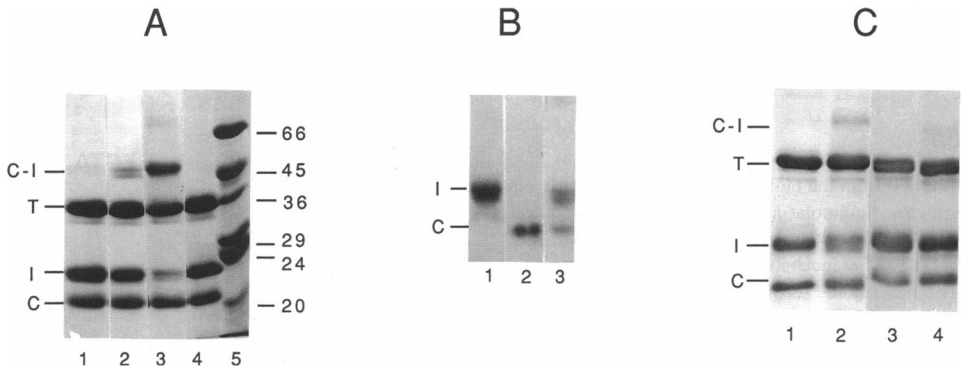

FIGURE 2 SDS-PAGE analysis of Nbs_2-catalyzed disulfide crosslinking between TnC and TnI. In panel *A*, lane 1 is untreated Tn complex; lane 2 is Tn treated with Nbs_2 in the presence of Ca^{2+} just before the addition of EGTA (see Fig. 1); lane 3 is material in lane 2 after addition of EGTA and allowed to react for ~30 min; lane 4 is material in lane 3 treated with DTT; lane 5 are molecular weight markers. In panel *B*, lanes 1 and 2 are TnI and TnC, respectively, excised from a polyacrylamide gel, then reelectrophoresed; lane 3 is the high molecular weight band that has been excised from lane 3, panel *A*, treated with DTT, then reelectrophoresed in the presence of DTT. In panel *C*, lane 1 is ternary Tn complexed composed of TnI, TnT, and TnC alkylated at Cys^{98}; lane 2 is the material in lane 1 treated with Nbs_2 first in the presence then in the absence of Ca^{2+} as described in the legend for Fig. 1; lane 3 is Tn complex composed of TnC, TnT, and TnI alkylated at Cys^{133}; lane 4 is the material in lane 3 treated with Nbs_2 first in the presence then in the absence of Ca^{2+} as described in the legend for Fig. 1. All samples were treated with excess iodoacetamide to block free sulfhydryls before SDS-PAGE.

TABLE 1 Ca^{2+}-dependent ATPase activity of a reconstituted system composed of S1, Tm, actin, and Tn that has been treated with varying amounts of Nbs_2 to induce varying extents of disulfide crosslinking*

	ATPase activity (s^{-1})		Ca^{2+} sensitivity‡
$[Nbs_2]/[Tn]$	$-Ca^{2+}$	$+Ca^{2+}$	(%)
0	0.05	0.24	80
0.2	0.10	0.21	52
0.4	0.17	0.22	23
0.8	0.20	0.20	0
1.0	0.22	0.22	0

* Concentrations of materials in the assay solution were: [S1] = 2 μM, [F-actin] = 3 μM, [Tm] = [Tn] = 0.5 μM, [EGTA] = 4 mM, or $[Ca^{2+}]$ = 0.1 mM. Temperature was 25°C.

‡ Defined as $[1 - ATPase(-Ca^{2+})/ATPase(+Ca^{2+})] \times 100$.

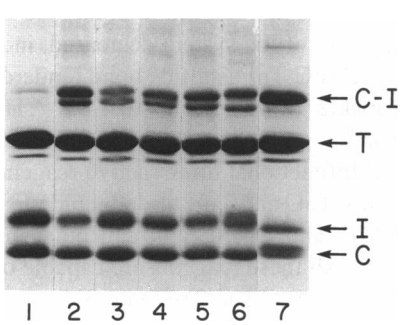

FIGURE 3 Crosslinking of TnC and TnI by thio-specific bifunctional reagents. Ternary Tn complex (lane 1) was treated with *o*-PDM (lane 2), *p*-PDM (lane 3), FNB (lane 4), BMME (lane 5), and DMSDS (lane 6) in the absence of Ca^{2+} as described in Materials and Methods. Lane 7 is Tn treated with Nbs_2 as described in the legend for Fig. 1.

modifies Cys^{133} of TnI because it is the only reactive sulfhydryl under this condition. One mol of TNB^- ion is released, giving rise to the observed rise in A_{412}; 2) addition of EGTA thereafter results in Cys^{98} of TnC becoming reactive. Crosslinking occurs via disulfide exchange; a second mol of TNB^- is released giving rise to the further increase in A_{412}.

It was noted that at the end of the reaction more TnC remains than TnI (Fig. 1 *A*, lane 3), whereas our reaction scheme would predict concommitant removal of TnC and TnI. This may be due to inaccuracies in the amounts of the added proteins, although the same result was obtained using purified instead of reconstituted Tn (data not shown). A more likely explanation is that Tn aggregates to a small extent and that Nbs_2 induces disulfide crosslinking between TnI subunits in the aggregates. It is clear, however, that the major reaction product is the disulfide-crosslinked TnC-TnI complex.

Aside from disulfide crosslinking, Dobrovol'sky et al. (1984) showed that FNB can crosslink the two Cys's, an observation that is confirmed here. We found further that these two Cys's can be crosslinked by four bismaleimide compounds whose maleimidyl groups are separated by a methyl ether group (BMME), phenyl group in the ortho configuration (*o*-PDM), phenyl group in the para configuration (*p*-PDM), and stilbene group (DMSDS). Except for BMME, the inter-maleimide linker groups of all these compounds are rigid. All of the crosslinking results suggest that the distance between these two Cys's is not fixed, and can span from 2 Å (to form a disulfide bond) to ~18 Å (to accommodate the stilbene group of DMSDS). It is interesting to note that our previous energy transfer studies also revealed some heterogeneity in the distance separating these two Cys's (Tao et al., 1989), suggesting that the protein regions containing these two Cys's possess considerable flexibility, a feature that may be required for the regulatory function of these proteins.

It was noted that whereas the disulfide crosslinked TnC-TnI complex comprises a single band, the bismaleimide crosslinked product is composed of two bands. Because it is known that the maleimide moiety is less specific than Nbs_2 for sulfhydryls, it is possible that the second (lower) band

corresponds to crosslinking between Cys^{133} of TnI and a non-Cys residue in TnC.

Our results show that the formation of a disulfide bond between Cys^{133} of TnI and Cys^{98} of TnC abolished the capacity of the Tn complex to regulate acto-S1 ATPase activity in a Ca^{2+}-dependent manner; specifically, Tn containing this crosslink is incapable of inhibiting acto-S1 ATPase in the absence of Ca^{2+}. We believe that this observation is relevant to the mechanism of thin filament-based regulation in the following manner: it has been proposed that activation of striated muscle contraction occurs when Ca^{2+} binds to the triggering sites of TnC (sites I and II), whereupon the affinity between TnC and TnI is increased, resulting in TnI detaching from actin. This in turn gives rise to the movement of Tm to a position in the thin filament that allows the cyclic interaction between myosin heads and actin to take place (El-Saleh et al., 1986).

Previous reports showing that upon the binding of Ca^{2+} to TnC, Cys^{133} of TnI moves away from Cys^{374} of actin (Tao et al., 1990), and towards Cys^{98} of TnC (Tao et al., 1989; Wang and Cheung, 1984) are in support of the above model. Our present work is also in support of this model because the disulfide crosslink between Cys^{133} of TnI and Cys^{98} of TnC can be expected to inhibit the movement of the Cys^{133} region of TnI away from TnC and towards actin when Ca^{2+} is removed; the crosslink, therefore, locks the thin filament in the activated state, giving rise to our observed constitutively active acto-S1 ATPase.

It should be noted that in the absence of the high resolution structure of TnI, the location of Cys^{133} in the TnI molecule is not known. We can conjecture, however, that it is in the vicinity of the so-called "inhibitory region" (residues 96–116), the region that contains the inhibitory peptide sequence (Syska et al., 1976), and most likely undergoes the Ca^{2+}-dependent switching between TnC and actin. This is based on our findings that Cys^{98} of TnC can be crosslinked to the inhibitory region of TnI via an attached photocrosslinker (Leszyk et al., 1987, 1988), and to Cys^{133} of TnI via a disulfide bond (this work). Taken together, our findings suggest that a portion of the TnI molecule comprising both the inhibitory and the Cys^{133} region undergoes switching between TnC and actin during Ca^{2+} activation of skeletal muscle contraction.

We thank Dr. Gale Strasburg for carrying out some of the initial crosslinking studies. We thank Drs. John Gergely and Zenon Grabarek for critical comments and reviewing the manuscript.

This work was supported by National Institutes of Health grant AR21673. Hye-Shin Park was a postdoctoral fellow of the American Heart Foundation, Massachusetts affiliate.

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