Cross-Linking of Rabbit Skeletal Muscle Troponin Subunits: Labeling of Cysteine-98 of Troponin C with 4-Maleimidobenzophenone and Analysis of Products Formed in the Binary Complex with Troponin T and the Ternary Complex with Troponins I and T†

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ABSTRACT: The sulfhydryl-specific, heterobifunctional, photoactivatable cross-linker 4-maleimidobenzophenone (BPMal) was used to study the interaction of rabbit skeletal muscle troponin subunits TnC, TnT, and TnI. TnC was labeled at Cys-98 by the maleimide moiety of BPMal and then mixed with either TnT alone or TnI plus TnT, in the presence of Ca^{2+}. Upon photolysis, TnI and/or TnT formed covalent cross-links with TnC. The cross-linked TnC–TnT heterodimer obtained from the binary complex was digested into progressively smaller cross-linked peptides that were purified by HPLC and then characterized by amino acid analysis and sequencing. An initial cross-linked CNBr fraction contained the expected peptide CB9 (residues 84–135) of TnC, plus CNBr peptides spanning residues 152–230 of TnT. Results from a peptic digest of the CNBr cross-linked fraction permitted the identification of residues 159–197 as the most highly cross-linked region in TnT. A final subtilisin digest yielded a heterogeneous cross-linked fraction, which suggested that an especially high degree of cross-links was formed in the vicinity of residues 175–178 (Met-Lys-Lys-Lys) of TnT. Although this region of TnT had previously been implicated in binding, we show here for the first time that it is close to Cys-98 of TnC. In an analogous study on the binary complex of TnC and TnI [Leszyk, J., Collins, J. H., Leavis, P. C., & Tao, T. (1987) *Biochemistry 26*, 7042–7047], we previously showed that Cys-98 of TnC was cross-linked mainly to CN4, the "inhibitory region", of TnI. In this report we analyze the cross-linked products from the complex of all three troponin subunits and find that CN4 (residues 96–116) of TnI and CB4 (residues 176–230) of TnT are the predominant cross-linked CNBr peptides in these two proteins. This indicates that the Cys-98 region of TnC interacts with TnI and TnT in a similar manner in both the ternary and binary complexes.

The interaction of actin and myosin that occurs during muscle contraction is regulated by changes in intracellular Ca^{2+} concentration. In vertebrate striated muscles the dominant regulatory system involves binding of Ca^{2+} to troponin in the thin filaments. Troponin is a complex of three different protein subunits: TnC[1] binds Ca^{2+}, TnI binds to actin and inhibits actin–myosin interaction, and TnT binds to tropomyosin. The amino acid sequences of all three rabbit fast skeletal muscle troponin subunits have been determined (Collins et al., 1973, 1977; Wilkinson & Grand, 1975; Pearlstone et al., 1977), and these proteins have served as models for extensive structure–function studies carried out in several laboratories [see Leavis and Gergely (1984) and Zot and Potter (1987) for reviews]. While much remains to be learned about the three-dimensional structure of rabbit skeletal muscle TnI and TnT, analysis of the TnC sequence (Collins et al., 1973) predicted the locations of four Ca^{2+}-binding sites, designated I–IV going from the amino to the carboxyl terminus of the protein. This prediction was confirmed by the more recently available crystal structures of chicken (Sundaralingam et al., 1985) and turkey (Herzberg & James, 1985) TnCs.

Many studies have focused on the mechanism of transmission of Ca^{2+}-induced structural changes from one thin filament protein to another (Leavis & Gergely, 1984; Zot & Potter, 1987). A key aspect of this problem is the elucidation of interactions among the thin filament proteins, particularly among the three troponin components. In the case of the binding of TnC to TnT or TnI, considerable information has been obtained from studies of the binding of various proteolytic fragments (Pearlstone & Smillie, 1978; Syska et al., 1976; Grabarek et al., 1981), from the reactivities of surface amino acids (Hitchcock, 1981; Hitchcock et al., 1981), and from various spectroscopic studies (Leavis et al., 1984). There appear to be several sites of interaction among the troponin subunits, and these sites seem to be confined to highly conserved regions of the molecules. Little is known, however, about the identities of the particular amino acid residues that make up these sites.

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[1] Abbreviations: TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; PITC, phenyl isothiocyanate; HPLC, high-performance liquid chromatography; TnC, troponin C; TnI, troponin I; TnT, troponin T; BPMal, 4-maleimidobenzophenone; BP-TnC, TnC labeled with BPMal; BP-TnC*T, cross-linked heterodimer of TnT and BP-TnC; BP-TnC*I, cross-linked heterodimer of TnI and BP-TnC.

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Of particular interest for this report are previous cross-linking studies carried out on native troponin which indicate some TnC–TnT contact, although the major cross-linked products obtained have been TnC–TnI and TnT–TnI (Hitchcock, 1975; Sutoh, 1980). In these studies, homobifunctional reagents that reacted with multiple Lys residues were used, yielding extremely complex mixtures of cross-linked species. More useful information on TnC–TnI (Chong et al., 1981; Dobrovl'sky et al., 1984; Leszyk et al., 1987) and TnT–TnI (Chong et al., 1982) interactions has been obtained from studies which used cross-linkers that react specifically with the far less numerous sulfhydryl groups in these proteins.

Tao et al. (1985, 1986a,b) demonstrated that BPMal, a thiol group specific, high-yield photochemical cross-linker, shows great potential as a probe of thin filament protein interactions. In particular, they showed that BP-TnC (labeled at Cys-98) forms binary complexes with either TnI or TnT, or a ternary complex with both TnI and TnT, and that photolysis produces the cross-linked heterodimers BP-TnC*I and/or BP-TnC*T. In a later report (Leszyk et al., 1987), we described the characterization of BP-TnC*I obtained from a binary complex. We showed that residues 107–111 of TnI, which are located in the "inhibitory region" of the molecule, are most highly cross-linked to Cys-98 of TnC. In the present study we characterize BP-TnC*T from the binary complex and also examine the two cross-linked heterodimers obtained from the ternary complex. This is the first reported use of a sulfhydryl-specific cross-linker for determining regions of interaction between TnC and TnT.

Experimental Procedures

Materials. All reagents were of the highest grade commercially available. Sequencer reagents were from Applied Biosystems. PTH-amino acid standards were from either Pierce or Applied Biosystems. Amino acid standards, PITC (for nonsequencer use), constant-boiling HCl, and CNBr were obtained from Pierce. Pepsin was obtained from Worthington. Subtilisin was obtained from Sigma. HPLC-grade water and TFA were obtained from Fisher. HPLC-grade acetonitrile was obtained from Burdick and Jackson. All other chemicals were obtained from Fisher.

Amino Acid Compositions and Sequence Analysis. Phenylthiocarbamylated acid hydrolysates of peptides were prepared by using the Waters "PICO-TAG" Work Station. PTC-amino acids were analyzed by reverse-phase HPLC (Heinrikson et al., 1984; Bidlingmeyer et al., 1984; Cohen et al., 1986) on a Waters "PICO-TAG" column, using the gradient elution system recommended by the manufacturer. Amino acid sequences were determined by using an Applied Biosystems Model 470A gas-phase protein sequencer as described by Hewick et al. (1981). PTH-amino acids obtained from the sequencer were also analyzed by reverse-phase HPLC, using a Waters Nova-Pak column and the gradient elution system described in Waters Associates Applications Brief M3500. The recovery of PTH-amino acids at each cycle was measured quantitatively. PTH-Ser and PTH-Thr were usually obtained in low yields and sometimes were not detectable at all; however, they could always be identified by the appearance of breakdown products which absorbed at 313 nm. PTH-Arg and PTH-His were also often recovered in low yields. A Waters HPLC system including two M510 pumps, a M721 system controller, a WISP 710A autoinjector, a temperature control module, a M440 dual-channel absorbance detector, and a M730 integrative recorder was used for both PTH (sequence) and PTC (composition) amino acid analyses. The detector was set at 254 nm to quantify PTC amino acids and the sum of 254 nm (to quantify PTH-amino acids) and 313 nm (for qualitative detection of breakdown products of PTH-Ser and PTH-Thr).

Preparative HPLC. All peptide mixtures were separated by reverse-phase HPLC using a 4.6 mm × 25 cm Vydac 218TP54 column. Two Waters M510 pumps, a Waters M680 controller, Waters M480 variable-wavelength absorbance detector, a Linear dual-channel recorder, and a Glenco SV-3 injector were used as our HPLC system. Solvent A was 0.1% TFA in acetonitrile/water (5:95 v/v), and solvent B was 0.1% TFA in acetonitrile/water (95:5 v/v). All separations were carried out by using a linear gradient at a flow rate of 1 mL/min. The eluent was monitored at 220 nm.

Preparation of Cross-Linked Peptides. Rabbit skeletal muscle troponin subunits were prepared by the method of Greaser and Gergely (1971). Labeling of TnC with BPMal and photo-cross-linking of BP-TnC with TnI and TnT were carried out as described previously (Tao et al., 1986b). The yields of the cross-linked products (20–40%), and their behavior on polyacrylamide gel electrophoresis, have been documented previously (Tao et al., 1986b). The heterodimer BP-TnC*T was purified from un-cross-linked and internally cross-linked proteins by ion-exchange chromatography on DEAE-Sephadex in 20 mM Tris buffer (pH 7.5) containing 2 mM EGTA, using a linear KCl gradient from 0.1 to 0.5 M. For preparation of cross-linked peptides, 57 nmol of BP-TnC*T was dissolved in 300 μL of 70% formic acid, 5 mg of CNBr was added, and digestion took place at room temperature for 20 h. The reaction mixture was diluted to 3 mL with water and dried at room temperature under a stream of nitrogen. The sample was then dissolved in 200 μL of 70% formic acid and applied to HPLC. The resulting cross-linked CNBr fraction (43 nmol) was dissolved in 300 μL of 5% formic acid, 35 μg of pepsin was added, digestion took place at room temperature for 6 h, and the digest was applied directly to HPLC. The cross-linked fraction (10 nmol) was dissolved in 200 μL of pH 8.1 buffer (0.1 M NH_4HCO_3 + 0.1 mM $CaCl_2$), 15 μg of subtilisin was added, digestion took place for 7.5 h at 37 °C, and the digest was applied directly to HPLC. The cross-linked heterodimers obtained from the ternary complex were separated from monomeric proteins by ion-exchange chromatography as described above. A mixture of approximately 2.5 nmol each of BP-TnC*I and BP-TnC*T was dissolved in 200 μL of 70% formic acid, 0.5 mg of CNBr was added, and digestion took place at room temperature for 19 h. The reaction mixture was then diluted to 2 mL with water, dried under nitrogen, redissolved in 100 μL of 70% formic acid, and applied to HPLC. As in our earlier study (Leszyk et al., 1987), cross-linked peptides were recognized in HPLC profiles of the various digests as broad, late-eluting peaks of relatively high absorbance (due to the benzophenone moiety) which yielded sequences around Cys-98 of TnC. Non-cross-linked peptide, which contained no such sequences, generally eluted as sharp peaks, and many of these could be identified by their amino acid compositions or by preliminary sequence analysis.

Results and Discussion

*BP-TnC*T from the Binary Complex.* HPLC of the CNBr digest of BP-TnC*T (Figure 1) yielded 12 fractions, which were subjected to amino acid analysis. Fractions 1–11 were identified as various CNBr peptides of TnC (Collins et al., 1977) and TnT (Pearlstone et al., 1977). Fraction 12 contained an unusually broad peak, and its amino acid composition resembled that expected from the sum of CB9 (residues 84–135) of TnC plus an equimolar mixture of the TnT peptides CB5 (residues 152–175) and CB4 (residues 176–230). Se-

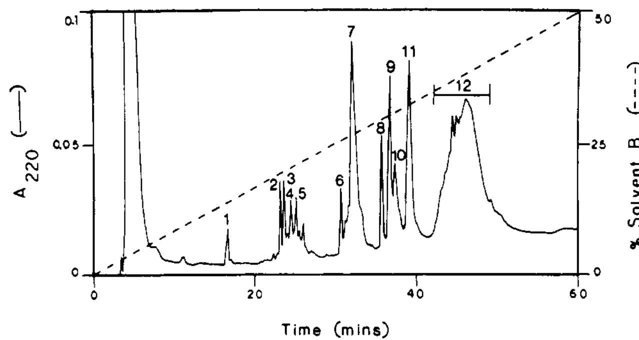

FIGURE 1: HPLC of a 3-nmol sample of the CNBr digest of BP-TnC*T obtained from the binary complex. The identities of the numbered fractions are as follows: 1, T-CB7; 2, T-CB6; 3, T-CB6; 4, T-CB5; 5, C-CB5; 6, C-CB4; 7, T-CB3; 8, C-CB7; 9, T-CB2; 10, T-CB2; 11, T-CB1; and 12, cross-linked peptides (see text). C-CB designates CNBr peptides of TnC (Collins et al., 1977), and T-CB designates CNBr peptides of TnT (Pearlstone et al., 1977). Some peptides eluted as double peaks, due to the homoserine to homoserine lactone conversion.

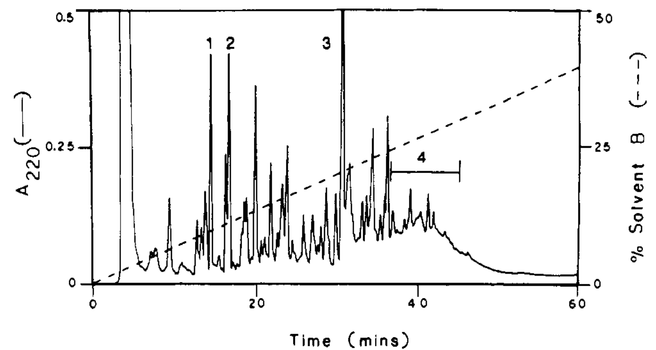

FIGURE 2: HPLC of a 40-nmol sample of the peptic digest of the CNBr cross-linked fraction from the binary complex (fraction 12 from Figure 1). Fractions 1, 2, and 3 were identified as TnT (155–158), TnC (109–114), and TnT (198–208), respectively, and fraction 4 was the cross-linked material.

quence analysis confirmed the presence of all three peptides and indicated that uncleaved CB5–CB4 of TnT was also a component of fraction 12. From quantitative analysis of recovered PTH-amino acids, it appeared that approximately 60% of the TnC-CB9 was cross-linked to TnT-CB4 and 40% was cross-linked to either TnT-CB5 or TnT-(CB5–CB4). It was difficult to accurately measure the relative amount of each peptide, due to complexities caused by variable yields, multiple sequences, and the carryover of PTH-amino acids from one cycle to the next. The sequencing of TnC-CB9 appeared to proceed normally, except for the absence of an identifiable PTH-amino acid corresponding to cross-linked Cys-98. All of the expected PTH-amino acids from TnT peptides were accounted for, and we were not able to identify from low sequencer yields any specific, individual cross-linked amino acids in TnT.

To investigate the possibility that fraction 12 of Figure 1 may contain un-cross-linked peptides, control HPLC runs (not shown) were carried out on CNBr digests of un-cross-linked TnC and TnT. We observed that CB5 and CB4 of TnT were well separated from fraction 12. The peptide CB5–CB4 was not recovered from un-cross-linked TnT, suggesting that CNBr cleavage at Met-175 was complete. TnC-CB9 could coelute with fraction 12 but would not be present in our sample since the starting material for CNBr digestion contained only purified, cross-linked heterodimer.

To more precisely locate the region of TnT that is close to Cys-98 of TnC, CNBr fraction 12 was further broken down with pepsin. HPLC of the peptic digest yielded a late-eluting, broad fraction of cross-linked material (Figure 2, fraction 4). Amino acid analysis of this fraction did not permit us to identify the constituent peptides. After sequence analysis, however, two short TnC peptides (residues 95–99 and 96–99, arising from partial cleavage at Leu-95) and approximately equal amounts of two overlapping TnT sequences (residues 159–183 and 176–197) were clearly identified. The 176–197 sequence represents an NH_2-terminal peptide from CB4, while the 159–183 sequence includes the COOH-terminal two-thirds of CB5 plus the NH_2-terminal region of CB4. The 159–183 sequence was obtained from a larger peptide, presumably spanning residues 159–197, which arose from peptic cleavage of CB5–CB4. Within the 159–183 sequence, both PTH-Met and PTH-Hse were obtained for Met-175, confirming that partial CNBr cleavage of CB5–CB4 had taken place. Therefore, residues in CB5, as well as those in CB4, were cross-linked to Cys-98 of TnC. The overall effect of peptic digestion was to reduce the span of cross-linked TnT peptides from 79 residues to 39, more precisely delineating the region of TnT that interacts with the Cys-98 region of TnC. Once again, however, we were unable to identify individual cross-linked residues in TnT.

Earlier binding studies on proteolytic fragments of rabbit skeletal muscle TnT and TnC have provided more limited information on the interactions between these two proteins. Grabarek et al. (1981) found that fragments of TnC containing residues 1–100 or residues 1–120 bind to whole TnT in the presence of Ca^{2+}. Weaker binding to TnT was found with TnC fragments 9–84 and 84–135. Pearlstone and Smillie (1978) showed that TnT fragments 1–205, 159–209, 176–230, 206–258, and 239–259 (from the carboxyl terminus) all bound to whole TnC. All of these fragments are derived, at least in part, from residues 159–259. This is significant because TnT fragments 159–209 and 206–258 were the ones that bound most strongly to TnC. Our results, which involve simultaneous fragmentation of both proteins, show that TnT residues 159–197 interact with Cys-98 of TnC. It is reasonable to then expect that TnT residues 198–259 must interact with some other region of TnC.

The cross-linked peptic fraction was further digested with the very nonspecific protease subtilisin. HPLC of the subtilitic digest (not shown) yielded an even broader and more heterogeneous cross-linked fraction. After sequence analysis of this fraction, the only identifiable (and by far the highest yielding) sequence was that of the TnC-derived peptide Ala-Glu-X-Phe (residues 96–99, where X is the cross-linked Cys-98). No specific sequence from TnT could be identified, although there was a high background of several PTH-amino acids in the first five cycles (after which no further PTH-amino acids were obtained). It is possible that cross-linked residues in TnT are fairly evenly distributed over residues 159–197, so that cleavage into very small peptides only increased the complexity of the cross-linked mixture. The final subtilisin digest apparently contained a large number of small, cross-linked peptides with overlapping sequences, making it impossible to identify any individual sequences from TnT. Despite this complexity, a relatively high background of PTH-Lys was noted in the sequencing of the cross-linked fraction. This observation, coupled with the reduced CNBr cleavage at Met-175, suggests that a high density of cross-links may be located in the vicinity of residues 175–178 of TnT, which has the sequence Met-Lys-Lys-Lys. In support of this idea, Hitchcock et al. (1981) found that, over the span of residues 159–197, Lys-176 to -178 of rabbit skeletal muscle TnT were the most reactive with acetic anhydride and that their reactivities were greatly reduced upon complexation with TnC and/or TnI.

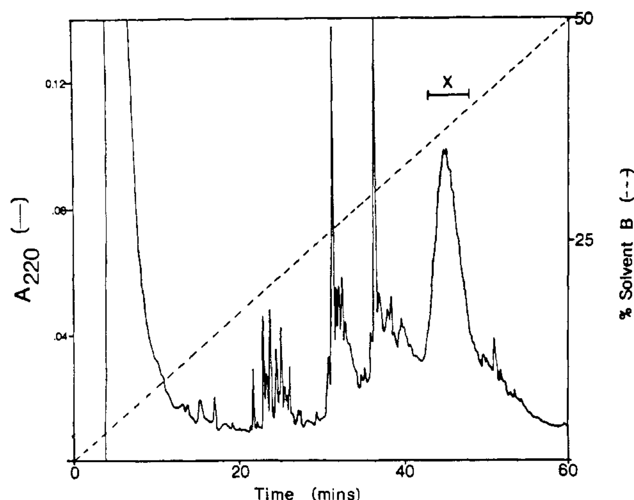

FIGURE 3: HPLC of a 2.5-nmol sample of the CNBr digest of the mixture of BP-TnC*I and BP-TnC*T obtained from the ternary complex. The cross-linked fraction indicated by an X was collected for sequence analysis.

The broad, heterogeneous nature of the cross-linked HPLC fractions is due to a population of cross-linked species that differ in their linkage sites on the TnT molecule. The benzophenone radical undoubtedly formed cross-links with several amino acid residues in TnT, and each amino acid side chain probably had several attachment sites. Furthermore, the formation of new carbon–carbon bonds from carbon–hydrogen bonds significantly increased the chirality of the cross-linked amino acids, resulting in even more diversity. As we have previously observed in analogous studies on BP-TnC*I (Leszyk et al., 1987), the cross-linked fractions became broader as they were further digested into smaller peptides, reflecting the increased contribution of cross-linked amino acid residues. The progressive removal of un-cross-linked residues left behind an increasingly heterogeneous mixture of smaller, cross-linked peptides whose variability could no longer be masked by the constant portions.

*BP-TnC*I and BP-TnC*T from the Ternary Complex.* Our studies were conducted on a mixture of BP-TnC*I and BP-TnC*T, free of monomeric (i.e., un-cross-linked and internally cross-linked) proteins. Attempts to separate the two heterodimers by ion-exchange and reverse-phase HPLC were unsuccessful. HPLC of the CNBr digest of the mixture yielded a chromatogram (Figure 3) that was very similar to that previously obtained from BP-TnC*I alone (Leszyk et al., 1987), although a close examination revealed the presence of additional peptides derived from TnT. Once again, there was a late-eluting, broad fraction (fraction X, Figure 3) that contained the cross-linked material. Sequence analysis of this fraction showed a strong TnC-CB9 sequence as well as those of TnI-CN4 and TNT-CB4. Significant amounts of TnI-CN4 (Leszyk et al., 1987) and TnT-CB4 (this report) were also found cross-linked to Cys-98 of TnC in the binary complexes. On the other hand, no TnT-CB5 sequence could be identified in fraction X of Figure 3, confirming previous observations (Leavis et al., 1984; Tao et al., 1986b) that TnC–TnT interactions differ between the binary and ternary complexes.

Leavis et al. (1984) found that complex formation with either TnT or TnI reduced the quenching of a fluorescent probe attached to Cys-98 of TnC, suggesting that TnT or TnI occupies the same binding site on TnC in the absence of the third subunit. Further support for this idea came from Hitchcock (1981), who found that the reactivities of the same Lys residues in TnC were reduced in both the TnC–TnT complex and the TnC–TnI complex. In this report we show for the first time that the regions in TnT or TnI that interact with the Cys-98 region of TnC in the binary complexes also interact in the presence of the third subunit. In other words, there appears to be little or no competition between TnI and TnT for a binding site on TnC in the vicinity of Cys-98.

Our results are consistent with the low-resolution crystal structure of troponin–tropomyosin (White et al., 1987), which shows TnT as an elongated molecule spanning 185 Å along the tropomyosin filament. The troponin complex contains a globular head region, which consists of TnC and TnI and lies near Cys-190 of tropomyosin, and a tail region that overlaps the head-to-tail junction of adjacent tropomyosin molecules in the filament. TnT is arranged such that its amino terminus forms the tail region of the complex and its carboxyl terminus is associated with the head region. Residues 159–197 of TnT, which we have shown to be close to Cys-98 of TnC in the binary complex, also interact with TnI (Pearlstone & Smillie, 1984) and so must reside in the globular head of the troponin complex. Circular dichroism measurements (Pearlstone & Smillie, 1978) indicate that this region of TnT has a largely (71%) random coil structure. It is known that residues 89–100 of TnC forms an α helix with an acidic face (Grabarek et al., 1981; Herzberg & James, 1985; Sundaralingam et al., 1985). This acidic region of TnC probably interacts with the basic 159–197 region of TnT and the basic CN4 region of TnI in an electrostatic manner.

Acknowledgments

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Supplementary Material Available

Tables I–III containing sequence data for cross-linked fractions (3 pages). Ordering information is given on any current masthead page.

References

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Chong, P. C. S., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 5071–5076.

Chong, P. C. S., & Hodges, R. S. (1982) *J. Biol. Chem. 257*, 11667–11672.

Cohen, S. A., Bidlingmeyer, B. A., & Tarvin, T. L. (1986) *Nature (London) 320*, 769–770.

Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., & Jackman, N. (1973) *FEBS Lett. 36*, 268–272.

Collins, J. H., Greaser, M. L., Potter, J. D., & Horn, M. J. (1977) *J. Biol. Chem. 252*, 6357–6362.

Dobrovl'sky A. B., Gusev, N. B., & Friedrich, P. (1984) *Biochim. Biophys. Acta 789*, 144–151.

Grabarek, Z., Drabikowski, W., Leavis, P. C., Rosenfeld, S. S., & Gergely, J. (1981) *J. Biol. Chem. 256*, 13121–13127.

Greaser, M., & Gergely, J. (1971) *J. Biol. Chem. 246*, 4226–4233.

Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65–74.

Herzberg, O., & James, M. (1985) *Nature (London) 313*, 653–659.

Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Hitchcock, S. E. (1975) *Biochemistry 14*, 5162–5167.

Hitchcock, S. E. (1981) *J. Mol. Biol. 147*, 153–173.

Hitchcock, S. E., Zimmerman, C. J., & Smalley, C. (1981) *J. Mol. Biol. 147*, 125-151.
Leavis, P. C., & Tao, T. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 724.
Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235-305.
Leavis, P. C., Gowell, E., & Tao, T. (1984) *Biochemistry 23*, 4156-4161.
Leszyk, J., Collins, J. H., Leavis, P. C., & Tao, T. (1987) *Biochemistry 26*, 7042-7047.
Pearlstone, J. R., & Smillie, L. B. (1978) *Can. J. Biochem. 56*, 521-527.
Pearlstone, J. R., & Smillie, L. B. (1984) *Can. J. Biochem. 63*, 212-218.
Pearlstone, J. R., Johnson, P., Carpenter, M. R., & Smillie, L. B. (1977) *J. Biol. Chem. 252*, 983-989.
Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985) *Science (Washington, D.C.) 227*, 945-948.
Sutoh, K. (1980) *Biochemistry 19*, 1977-1983.
Syska, H., Wilkinson, J. M., Grand, R. J. A., & Perry, S. V. (1976) *Biochem. J. 153*, 375-387.
Tao, T., Lamkin, M., & Scheiner, C. J. (1985) *Arch. Biochem. Biophys. 240*, 627-634.
Tao, T., Gergely, J., & Leavis, P. (1986a) *Biophys. J. 49*, 142-143.
Tao, T., Scheiner, C., & Lamkin, M. (1986b) *Biochemistry 25*, 7633-7639.
White, S. P., Cohen, C., & Phillips, G. N. (1987) *Nature (London) 325*, 826-828.
Zot, A. S., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535-559.

Cooperative Turning on of Myosin Subfragment 1 Adenosinetriphosphatase Activity by the Troponin-Tropomyosin-Actin Complex

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ABSTRACT: In the field of muscle regulation, there is still controversy as to whether Ca^{2+}, alone, is able to shift muscle from the relaxed to the fully active state or whether cross-bridge binding also contributes to turning on muscle contraction. Our previous studies on the binding of myosin subfragment 1 (S-1) to the troponin-tropomyosin-actin complex (regulated actin) in the absence of ATP suggested that, even in Ca^{2+}, the binding of rigor cross-bridges is necessary to turn on regulated actin fully. In the present study, we demonstrate that this is also the case for the turning on of the acto·S-1 ATPase activity. By itself, Ca^{2+} does not fully turn on the acto·S-1 ATPase activity; at low actin concentration, there is almost a 10-fold increase in ATPase activity when the regulated actin is fully turned on by the binding of rigor cross-bridges in the presence of Ca^{2+}. This large increase in ATPase activity does not occur because the binding of S-1·ATP to actin is increased; the binding of S-1·ATP is almost the same to maximally turned-off and maximally turned-on regulated actin. The increase in ATPase activity occurs because of a marked increase in the rate of P_i release so that when the regulated actin is fully turned on, P_i release becomes so rapid that the rate-limiting step precedes the P_i release step. These results suggest that, while Ca^{2+}, alone, does not fully turn on the regulated actin filament in solution, the binding of rigor cross-bridges can turn it on fully. If force-producing cross-bridges play the same role in vivo as rigor cross-bridges in vitro, there may be a synergistic effect of Ca^{2+} and cross-bridge binding in turning on muscle contraction which could greatly sharpen the response of the muscle fiber to Ca^{2+}.

The protein complex troponin-tropomyosin is responsible for regulating the contraction and relaxation of skeletal muscle (Weber & Murray, 1973; Ebashi, 1980). Muscle contraction occurs only when Ca^{2+} binds to this complex. In solution, the troponin-tropomyosin complex causes about 95% inhibition of the acto-S-1[1] ATPase activity in the absence of Ca^{2+} (Ebashi & Kodama, 1966; Hartshorne & Mueller, 1967). Since this inhibition occurs without troponin-tropomyosin, significantly affecting the binding of S-1·ATP to actin, we proposed that troponin-tropomyosin inhibits the acto-S-1 ATPase activity by blocking the release of P_i (Chalovich et al., 1981, 1983), rather than the binding of S-1·ATP to actin as was suggested by the steric blocking model.

In addition to affecting the acto-S-1 ATPase activity, troponin-tropomyosin confers cooperativity on the binding of S-1·ADP to actin in the absence of ATP (Greene & Eisenberg, 1980b). In the absence of both ATP and Ca^{2+}, troponin-tropomyosin markedly inhibits the binding of S-1·ADP to actin at low levels of saturation of the actin with S-1·ADP. However, at saturation levels greater than 50%, the binding strength

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMM, heavy meromyosin; NEM, *N*-ethylmaleimide; S-1, myosin subfragment 1; SH-1, the most reactive sulfhydryl group in S-1.

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