Direct Evidence for the Calcium-Induced Change in the Quaternary Structure of Troponin in Situ. Millisecond Cross-Linking of Troponin Components by a Photosensitive Heterobifunctional Reagent†

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ABSTRACT: Flash irradiation of the reconstituted troponin or thin filament complex in which one of their components, troponin C, was modified with a heterobifunctional photosensitive reagent before reconstitution of the troponin complex resulted in the formation of cross-links between troponin C and other components in contact with it. Quantitative analysis of the cross-linked products by gel electrophoresis has revealed interesting features of the quaternary structure of troponin. When the reconstituted troponin was photo-cross-linked with a xenon flash, an appreciable amount of cross-linking was detected between troponin C and troponin I and also between troponin C and troponin T. No effect of calcium on the cross-linking could be detected. This arrangement of components was found to change when troponin was complexed with F-actin–tropomyosin. The arrangement of troponin components in the thin filament complex was sensitive to calcium and magnesium; maximum cross-linking of troponin C and troponin I was observed when the thin filament was cross-linked in the presence of calcium and magnesium, while an appreciable decrease in the extent of the cross-linking was detected when calcium alone or calcium and magnesium were removed from the cross-linking medium. The cross-linking of troponin C and troponin T remained marginal irrespective of the concentration of calcium and magnesium.

Troponin, a regulatory protein in the vertebrate skeletal muscle, consists of three components, troponin C (TnC), troponin I (TnI), and troponin T (TnT).[1] TnC is the calcium binding protein, TnI inhibits actin–myosin interaction in the presence and absence of calcium, and TnT binds to tropomyosin. The interaction among these three components of troponin and that among the major components of the thin filament, that is, actin, tropomyosin, TnT, TnI, and TnC, have been extensively studied in various laboratories to understand the molecular mechanism of troponin action to produce the calcium-sensitive relaxation of muscle (van Eerd & Kawasaki, 1973; Hitchcock et al., 1973; Margossian & Cohen, 1973; Greaser & Gergely, 1973; Potter & Gergely, 1974; Mani et al., 1974; Ohnishi et al., 1975; Hitchcock, 1975a,b; Syska et al., 1976; Moir et al., 1977; Johnson et al., 1978; Horwitz et al., 1979). Mainly based on the binding studies, Potter & Gergely (1974) have proposed a model to show how the components are arranged in the thin filament and how calcium induces change in their arrangement. Similar models have also been proposed by Hitchcock et al. (1973) and Margossian & Cohen (1973).

To test their models, it is necessary to learn the arrangement of the major components of the thin filament in situ, in the presence and absence of calcium. Several chemical and physicochemical techniques have been developed in recent years to investigate the topology of complex, multicomponent systems [see reviews by Peters & Richards (1977) and Fairclough & Cantor (1978)]. Among them, the chemical cross-linking technique has enjoyed wide application in studies of a variety of biological phenomena, including studies of the topology of troponin components in the presence and absence of calcium by the use of imido ester and acyl azide cross-linkers (Hitchcock, 1975a; Hitchcock & Lutter, 1975). Although diimido ester cross-linkers such as dimethyl suberimidate are most popular for cross-linking protein complexes, it is sometimes difficult to assign cross-linked products especially when several kinds of components are present in the system. One way to facilitate the assignment of the cross-linked products is to use cleavable cross-linkers and diagonal gel electrophoresis as described by Sommer & Traut (1975) for the topological studies of ribosomal proteins and also by Wang & Richards (1974) for erythrocyte membrane proteins. Another way is to use stepwise cross-linkers as described by Ji (1977) for studies of the interaction between lectins and their receptors in erythrocyte membrane and by Erecinska et al. (1975) for cytochrome *c* interaction with mitochondrial membrane.

Several laboratories have reported the synthesis of stepwise cross-linkers carrying the aryl azide group on one end (Trommer & Hendrick, 1973; Hixon & Hixon, 1975; Kiem & Ji, 1977; Ji, 1977; Lewis et al., 1977). The main advantages of using the aryl azide group in a cross-linker are as follows (Bayley & Knowles, 1977). First, the aryl azide is chemically inert unless it is photolyzed at a wavelength of less than 350 nm. Second, the photolyzed product of the aryl azide, nitrene, is extremely reactive and can react with C–H bonds on the protein surface in a nonspecific fashion. Thus, the proper arrangement of two functional groups is not a requirement for the cross-linking. The third advantage is that the lifetime of nitrene is very short (<1 ms) in an aqueous solution (Reiser et al., 1971) and the cross-linking reaction can be completed within a few milliseconds when aryl azides are photolyzed by a flash of millisecond duration. Since the time scale required to unfold the polypeptide chain of a protein is supposed to be longer than that needed for the completion of the photo-cross-linking reaction (Matheson et al., 1977), the inherent difficulty in the chemical cross-linking technique, that is,

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[1] Abbreviations used: TnC, troponin C; TnI, troponin I; TnT, troponin T; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; ABI, methyl 4-azidobenzimidate; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; $NaDodSO_4$, sodium dodecyl sulfate.

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undesirable changes in protein conformation *during* the cross-linking reaction, can be greatly reduced by the millisecond cross-linking technique.

In the present study, the calcium binding component of troponin (TnC) was modified with a stepwise cross-linker, methyl 4-azidobenzimidate (ABI) and then troponin was reconstituted by using the ABI-modified TnC. ABI carries an aryl azide on one end and an imido ester on the other, and the two groups span ~5 Å (Ji, 1977; Lewis et al., 1977). Since only one component of troponin (TnC) carries cross-linkers, the cross-linked products formed by the flash photolysis of aryl azides necessarily contain TnC. Thus, the number of possible pairs of cross-linking is greatly reduced and the assignment of the cross-linked products becomes much easier. To facilitate the assignment, we labeled a component of troponin with the fluorescent dye *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) (Machida et al., 1975). By scanning fluorescent intensities along gels after electrophoresis in the presence of $NaDodSO_4$, we can only quantitate those species containing the DACM-labeled component.

Quantitative analysis of fluorescence profiles of $NaDodSO_4$ gels of the reconstituted troponin and thin filament complex irradiated by a xenon flash has revealed intersubunit interactions of TnC with other components. When troponin was free in solution, TnC was cross-linked with TnI and TnT, indicating that TnC is in contact with TnI and TnT. No effect of calcium on the cross-linking could be detected. When the troponin was complexed with F-actin–tropomyosin, TnC was again cross-linked with TnI and TnT. In this case, however, an effect of calcium and magnesium on the cross-linking of TnC and TnI was observed, suggesting that these divalent cations induced changes in the quaternary structure of troponin in the thin filament complex.

Materials and Methods

Reagents. All chemicals were reagent grade. DACM was kindly supplied by Dr. Yamamoto, Juntendo University, Tokyo, Japan.

Preparations of Proteins. Troponin and its three components, TnT, TnI, and TnC, were prepared according to Ebashi et al. (1971) and Ebashi (1974). The preparations of TnT and TnC were homogeneous when characterized by $NaDodSO_4$ gel electrophoresis. However, the TnI preparations showed proteolytic cleavage frequently; therefore, the following method including an acidification procedure (Willkinson, 1974) was employed for its preparation. Fresh troponin (4 mg/mL was dialyzed against 1 M KCl, 0.1 N HCl, and 4 mM 2-mercaptoethanol at 4 °C. The resulting turbid solution was centrifuged at 100000*g* for 1 h, and the supernatant was exhaustively dialyzed against 0.01 N HCl and 4 mM 2-mercaptoethanol. Then the dialysis solution was changed to 40 mM phosphate and 4 mM 2-mercaptoethanol (pH 6.4) containing 6 M urea. The 6 M urea was passed through a mixed column of Amberlite IR-120 and IRA-400 just before its use. The protein solution was passed through SP-Sephadex C-50 equilibrated with the same solvent. After the column was thoroughly washed with the solvent, TnI–TnT complex was eluted with 6 M urea, 0.5 M KCl, 40 mM phosphate, and 4 mM 2-mercaptoethanol (pH 6.4) (Wakabayashi et al., 1975). The resulting TnI–TnT complex was then dialyzed against 6 M urea, 0.1 M KCl, 2 mM $NaHCO_3$, and 4 mM 2-mercaptoethanol and passed through SP-Sephadex C-50 equilibrated with the solvent. TnI was first eluted and then TnT by increasing the KCl concentration to 0.2 M. TnT and TnI thus obtained were homogeneous when checked by $NaDodSO_4$ gel electrophoresis. The proteins were stored at −20 °C in solvents containing 6 M urea. The acidification procedure damaged neither TnI nor TnT, since full activity to confer calcium sensitivity to actomyosin ATPase was regained when the troponin reconstituted from the acid-treated TnI–TnT complex and TnC isolated without the acidification procedure was mixed with tropomyosin and desensitized actomyosin so that the molar ratio of actin, tropomyosin, and troponin was 10:1:1.

Desensitized actomyosin was prepared according to Syska et al. (1976). In the preparation, the major components were actin and myosin with some minor components such as C-protein and α-actinin. Neither troponin nor tropomyosin could be detected by $NaDodSO_4$ gel electrophoresis even when a large amount of protein was loaded on gels, consistent with the observation that calcium did not affect the ATPase activity of the actomyosin preparations. The weight ratio of actin to myosin in the preparations estimated by the intensities of Coomassie staining was 1:2.4, and ~25% (weight) of the total protein in the actomyosin preparations was actin.

Actin was prepared according to Spudich & Watt (1971) and tropomyosin according to Wakabayashi et al. (1975).

Labeling of TnC and TnI with DACM. TnC (1 to 2 mg/mL) was labeled with DACM in 5 mM imidazole (pH 7.0) for 10 min at 0 °C by using a threefold molar excess of DACM to TnC. The reaction was terminated by the addition of 0.01 volume of 100 mM 2-mercaptoethanol. TnC labeled with DACM is denoted as TnC(DACM).

Whole troponin was used to label TnI, since isolated TnI easily aggregated in the absence of a reducing reagent which must be removed from the solvent before the DACM labeling of TnI. Whole troponin (5 mg/mL) in 5 mM imidazole (pH 7.0) was labeled with DACM by using a 1.2 molar excess to troponin. The reaction was allowed to proceed for 1 h at 0 °C and was terminated by the addition of 2-mercaptoethanol. Under the labeling conditions, only TnI was labeled with DACM. DACM-labeled TnI was then isolated and purified as described above. TnI labeled with DACM is denoted as TnI(DACM).

Reconstituted troponin with DACM-labeled TnI or TnC regained full activity to confer calcium sensitivity to actomyosin ATPase under the conditions described below.

Modification of TnC with ABI. TnC (1 to 2 mg/mL) in 5 mM imidazole (pH 7.0) was mixed with an equal volume of ABI (3 to 4 mg/mL) in 0.4 M $NaHCO_3$–Na_2CO_3 (pH 9.5), and the amidination reaction was allowed to proceed for 2 to 3 h at 25 °C. The reaction was stopped by the addition of 0.05 volume of 1 M lysine. The resulting solution was dialyzed against 20 mM imidazole (pH 7.0) exhaustively, and then the dialysis buffer was changed to 6 M urea and 10 mM imidazole (pH 7.0). All procedures were carried out in the dark or under a red safety light. TnC modified with ABI is denoted as TnC(ABI).

Formation of the TnC–TnI and TnC–TnT Complexes and Reconstitution of Troponin. TnC–TnI complex, TnC–TnT complex, and reconstituted troponin were formed by placing equimolar amounts of each component together in 6 M urea and then dialyzing the mixture successively against 0.5, 0.25, and 0.1 M KCl containing 10 mM imidazole (pH 7.0). The resulting clear solution was centrifuged at 100000*g* for 2 h before use. When TnC(DACM–ABI) was used, all procedures were carried out in the dark or under a red safety light.

Reconstitution of the Thin Filament Complex. F-Actin was complexed with tropomyosin in a 7:1 molar ratio in 0.1 M KCl and 10 mM imidazole (pH 7.0). A 0.7-mol amount of the reconstituted troponin per mol of tropomyosin was then mixed

with the F-actin–tropomyosin complex.

Measurement of ATPase Activity. For ATPase activity measurement, a mixture of desensitized actomyosin (0.2 mg/mL), tropomyosin (10 μg/mL), and troponin (10 μg/mL) in 50 mM KCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) was used. In the mixture, the molar ratio of actin, tropomyosin, and troponin was about 10:1:1. The ATPase reaction was initiated by the addition of 0.1 volume of 10 mM ATP, allowed to proceed for 5 min at 25 °C, and then terminated by the addition of an equal volume of 20% trichloroacetic acid. The phosphate liberation was measured by the Fiske–SubbaRow method (Fiske & SubbaRow, 1925).

Flash Photolysis Apparatus. A flash photolysis apparatus was constructed by using a xenon flash tube (Fuji-koken, JLX-10S) which was fired at 1.2 kV and discharged in 1 ms. The output power was 1 kJ. The photolysis cell was constructed from two quartz tubes with diameters of 20 and 24 mm, respectively. The sample compartment was a cylindrical annulus of 1-mm path length, 35 mm long. A similar apparatus was previously described by Matheson et al. (1977).

Cross-Linking Reaction. Three milliliters of sample solution containing 0.2 mg/mL ABI-modified TnC was placed in the cylindrical annulus of the photolysis cell. The cross-linking reaction was initiated by discharging the xenon flash tube. The procedures were carried out in the dark or under a red safety light except for the irradiation step. The temperature was 25 °C.

Synthesis of the Cross-Linking Reagent. Methyl 4-azidobenzimidate was synthesized according to Ji (1977) and Lewis et al. (1977). The purity of the reagent was checked by thin-layer chromatography and infrared spectroscopy. It was stored in a vacuum desiccator on P_2O_5 at 4 °C. Under the conditions no appreciable decomposition of the reagent was observed for several months.

Scanning of Fluorescent Intensities along $NaDodSO_4$ Gels. A fluorescence gel scanner was constructed according to Yamamoto et al. (1978). The distribution of DACM-labeled protein along gels was measured directly in glass tubes after gel electrophoresis. Protein concentration was kept in the range where fluorescent intensity is proportional to the amount of DACM present (Yamamoto et al., 1978).

For quantitation, the same gel was scanned 3 times. The area under the curve was cut, weighed, and averaged.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out according to Weber & Osborn (1969) by employing 50 mM Tris-HCl buffer. After the gels were stained with Coomassie Blue, they were scanned with a commercial densitometer (Fuji-Riken, FD-IV) and quantitation was carried out as described above. Scanning of fluorescent intensity was carried out without Coomassie staining as shown above. Apparent molecular weights of the cross-linked products were estimated by using the following marker proteins: C-protein (M_r 140 000), subfragment 1 of myosin (M_r 90 000), bovine serum albumin (M_r 68 000), catalase (M_r 58 000), actin (M_r 42 500), TnT (M_r 30 500), TnI (M_r 21 000), TnC (M_r 18 000).

Miscellaneous Procedures. The concentration of proteins was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

The content of free amines was determined by the method of Habeeb (1966).

Results

Effect of DACM and ABI Modifications of TnC on the Biological Activity of the Reconstituted Troponin. TnC in 5 mM imidazole (pH 7.0) was first labeled with a threefold molar excess of DACM which is known to label SH groups of a protein at neutral pH (Yamamoto et al., 1977) and then modified with ABI in 0.2 M $NaHCO_3$–Na_2CO_3 (pH 9.5) in the presence of a large excess of the cross-linker. Alkaline pH was used for the modification reaction since lower pH would lead to undesirable side reactions as shown by Browne & Kent (1975). Titration of free amino groups by the TNBS (trinitrobenzenesulfonate) method (Habeeb, 1966) indicated that about one-third of the total amino groups of TnC [~3 out of 10 amino groups (Collins et al., 1977)] were modified with ABI when 1 mg/mL TnC was reacted with 2 mg/mL ABI for 2 h (see experiment 3 in Table II).

Table I: Effect of DACM and ABI Modifications of TnC on Calcium Sensitivity[a]

	0.1 mM $CaCl_2$	0.5 mM EGTA	calcium sensitivity[b] (%)
no troponin	0.420[c]	0.410[c]	2
control troponin	0.432	0.189	56
TnC-TnI-TnT	0.402	0.172	57
TnC(DACM-ABI)-TnI-TnT[d]	0.403	0.136	66

[a] Assay conditions were 50 mM KCl, 10 mM imidazole, 2 mM $MgCl_2$, and 1 mM ATP (pH 7.0) in the presence of 0.1 mM $CaCl_2$ or 0.5 mM EGTA at 25 °C. Each assay contained 0.2 mg of desensitized actomyosin and 10 μg of tropomyosin in 1 mL. When troponin was used, 10 μg was added to the mixture. The ATPase reaction was started by the addition of 0.1 mL of 10 mM ATP and terminated by the addition of an equal volume of 20% trichloroacetic acid after 5 min. [b] Calcium sensitivity = [1 − (EGTA ATPase/Ca^{2+} ATPase)] × 100 (%). [c] Micromoles of P_i per milligram of actomyosin per minute. [d] Conditions for the ABI modification of TnC: TnC, 1 mg/mL; ABI, 2 mg/mL; reaction time, 2 h; temperature, 25 °C.

Troponin complex was reconstituted from TnI, TnT, and the doubly modified TnC and then its activity to confer calcium sensitivity to actomyosin ATPase was assayed to check if these modification procedures induce any conformational change in the protein and affect interactions of TnC with other components of troponin. A typical result is presented in Table I. The calcium sensitivity of troponin which has not experienced any dissociation or modification procedures (control troponin) is 56%, while that of the reconstituted troponin without any modification is 57%. When the DACM- and ABI-modified TnC [TnC(DACM–ABI)] was used for the reconstitution, the calcium sensitivity is 66%. Experimental error in calculating the calcium sensitivity is ~10%. It must be noted here that the molar ratio of actin, tropomyosin, and troponin is 10:1:1 in the assay mixture and the content of tropomyosin and troponin is less than that in the original thin filament. The above observation leads to the conclusion that neither DACM nor ABI modification induces significant structural changes in TnC or in reconstituted troponin.

Cross-Linking of TnC(DACM–ABI)–TnI Complex or TnC(DACM–ABI)–TnT Complex. TnC(DACM–ABI)–TnI complex was cross-linked by flash photolysis of aryl azides on TnC in 0.1 M KCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA. The resulting solutions were electrophoresed in the presence of $NaDodSO_4$ to analyze the cross-linked products. When gels were stained with Coomassie Blue, it was observed that the photo-cross-linking caused a decrease in the band intensities of TnC (M_r 18 000) and TnI (M_r 21 000) to the same extent, with a concomitant increase in the intensity of a new band whose apparent molecular weight is 38 000, as shown in Figure 1. Note that no cross-linking was observed between TnC and TnI unless the complex was irradiated by a xenon flash, as

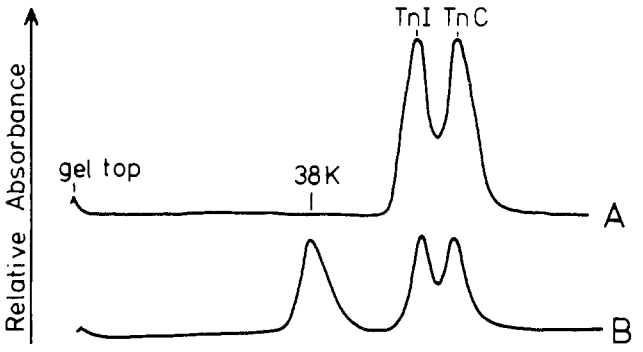

FIGURE 1: Cross-linking of TnC(DACM-ABI)-TnI complex. Densitometer traces of $NaDodSO_4$-acrylamide gels (10%) stained with Coomassie Blue. (Trace A) Before irradiation with a xenon flash; (trace B) after irradiation. Conditions for the ABI modification of TnC: TnC, 1 mg/mL; ABI, 2 mg/mL; solvent, 0.2 M $NaHCO_3$-Na_2CO_3 (pH 9.5); reaction time, 2 h; temperature, 25 °C. Conditions for the photo-cross-linking by a xenon flash: solvent, 0.1 M KCl, 10 mM imidazole, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (pH 7.0); temperature, 25 °C.

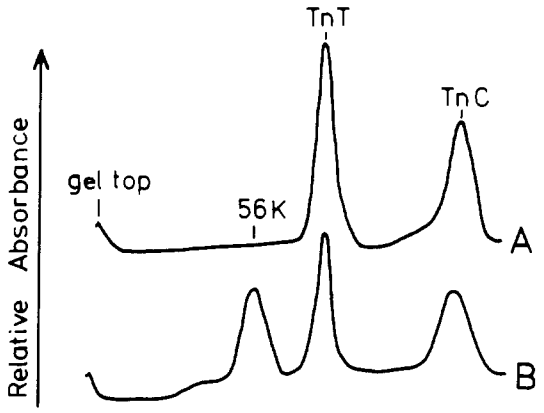

FIGURE 2: Cross-linking of TnC(DACM-ABI)-TnT complex. Densitometer traces of $NaDodSO_4$-acrylamide gels (10%) stained with Coomassie Blue. (Trace A) Before irradiation with a xenon flash; (trace B) after irradiation. Conditions for the ABI modification of TnC and those for the photo-cross-linking were the same as those in Figure 1.

shown in trace A in Figure 1. It must be also noted that no appreciable cross-linking of troponin components was observed when troponin without carrying aryl azides was used for the photo-cross-linking. The 38K band is assigned as the cross-linked product of TnC and TnI. The intensity of the 38K band is independent of calcium.

For TnC(DACM-ABI)-TnT complex, a cross-linked product with an apparent molecular weight of 56 000 was observed with a concomitant loss of intensity in the TnC and TnT (M_r 30 500) bands, as shown in Figure 2. The 56K species is the cross-linked product of TnC and TnT. No appreciable difference was detected in the cross-linking pattern in the presence and absence of calcium.

Cross-Linking of Reconstituted Troponin. Troponin reconstituted from TnC(DACM-ABI), TnI, and TnT was irradiated with a xenon flash to form cross-links in 0.1 M KCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) in the presence and absence of calcium. The cross-linked product of TnC and TnI would overlap with the TnT band on $NaDodSO_4$ gels, considering their apparent molecular weights. Therefore, instead of Coomassie Blue staining, the fluorescent intensity of DACM covalently linked to a component of troponin was scanned along gels to quantitate the cross-linked products later on.

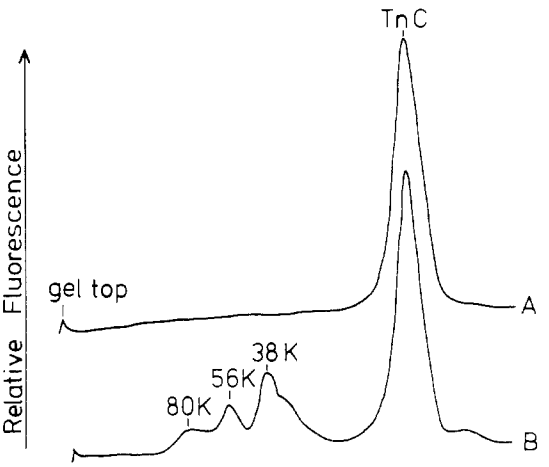

FIGURE 3: Cross-linking of the reconstituted troponin, TnC(DACM-ABI)-TnI-TnT. Traces of fluorescence scanning along $NaDodSO_4$-acrylamide gels (10%) of the reconstituted troponin complex before (trace A) and after (trace B) the photo-cross-linking reaction with a xenon flash. The fluorescent intensity of DACM covalently linked to TnC was scanned by the fluorescence gel scanner as described under Materials and Methods. Conditons for the ABI modification of TnC and those for the photo-cross-linking were the same as those in Figure 1.

Figure 3 shows the result of fluorescence scanning of $NaDodSO_4$ gels of the reconstituted troponin [TnC(DACM-ABI)-TnI-TnT] cross-linked with a flash irradiation in the presence of 0.1 mM $CaCl_2$. Before the irradiation, only TnC is observed on the gel, as shown in trace A of Figure 3, while three peaks of cross-linked products with apparent molecular weights of 38 000, 56 000, and 80 000 are detected after irradiation, as shown in trace B of Figure 3. The 38K peak has a shoulder on its leading edge, whereas the 56K and 80K peaks are symmetrical. Quantitative analysis of the fluorescent intensity profile revealed that 20% of the total fluorescent intensity is present in the 38K peak and 10% in the 56K peak. The 80K peak contains only a small percent of the total intensity. When the reconstituted troponin was cross-linked in the absence of calcium (in the presence of 1 mM EGTA), the same fluorescence profile as seen in Figure 3 was obtained, implying that the quaternary structure of troponin free in solution is rather insensitive to calcium.

The 38K peak in trace B of Figure 3 is assigned as the cross-linked product of TnC and TnI and the 56K peak as that of TnC and TnT, considering the fact that their apparent molecular weights coincide with those estimated before for TnC-TnI and TnC-TnT complex. The 80K material would be the cross-linked product of TnC-TnI-TnT.

TnC(ABI)-TnI(DACM)-TnT complex was photo-cross-linked in place of TnC(DACM-ABI)-TnI-TnT complex to fortify the above assignment. The distribution of DACM-labeled TnI along gels was shown in Figure 4. Before irradiation by a xenon flash, only TnI was detected on the gel. Irradiation of the TnC(ABI)-TnI(DACM)-TnT complex yielded a new peak with an apparent molecular weight of 38 000, as shown in trace B of Figure 4. This peak has a shoulder on its leading edge, as observed before in Figure 3. About 20% of the total intensity is present in the 38K peak. Thus, the 38K peak with a shoulder on its leading edge (seen in Figure 3) is unequivocally assigned as the cross-linked product of TnC and TnI.

Cross-Linking of Reconstituted Troponin Incorporated in the Thin Filament Complex. The reconstituted troponin, TnC(DACM-ABI)-TnI-TnT, was mixed with the F-actin-tropomyosin complex to form reconstituted thin filament. The

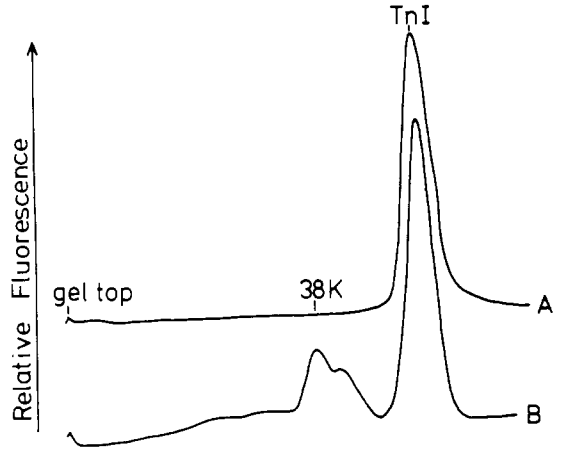

FIGURE 4: Cross-linking of the reconstituted troponin, TnC(ABI)–TnI(DACM)–TnT. Traces of fluorescence scanning of NaDodSO$_4$–acrylamide gels (10%) before (trace A) and after (trace B) the photo-cross-linking reaction with a xenon flash. Conditions for the ABI modification of TnC and those for the photo-cross-linking reaction were the same as those in Figure 1. Notice that the fluorescent dye was covalently linked to TnI while ABI was covalently linked to TnC.

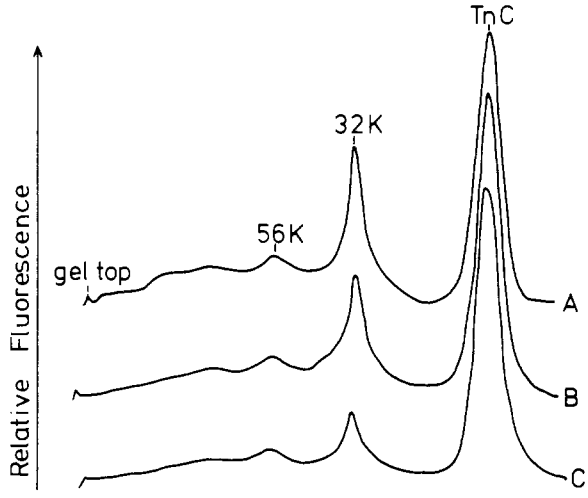

FIGURE 5: Cross-linking of the TnC(DACM–ABI)–TnI–TnT complex incorporated in the thin filament. Traces of fluorescence gel scanning of NaDodSO$_4$–acrylamide gels (10%) of the photo-cross-linked thin filament containing the reconstituted troponin complex, TnC-(DACM–ABI)–TnI–TnT. Conditions for irradiation with a xenon flash: 0.1 M KCl, 10 mM imidazole, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.0 (trace A); 0.1 M KCl, 10 mM imidazole, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.0 (trace B); 0.1 M KCl, 10 mM imidazole, and 1 mM EDTA, pH 7.0 (trace C). Conditions for the ABI modification of TnC were the same as those in Figure 1. Photo-cross-linking reactions were carried out at 25 °C.

thin filament complex was then irradiated by a xenon flash in 0.1 M KCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA to form cross-links between the ABI-modified TnC and other components in contact with TnC. After the irradiation, one major and one minor fluorescent peaks of cross-linked products appear on NaDodSO$_4$ gels, as observed in Figure 5. The apparent molecular weight of the major peak is 32 000 and that of the minor peak is 56 000. Differences between fluorescence profiles in Figures 3 and 5 are easily recognized. First, the 56K peak diminishes its intensity when the reconstituted troponin is complexed with F-actin–tropomyosin. Only a small percent of the total fluorescent intensity is present in the 56K peak in Figure 5 while 10% is in the 56K peak in Figure 3. Second, the peak corresponding to the cross-linked product of TnC and TnI (seen as the 38K peak in Figure 3) now

Table II: Effect of Calcium and Magnesium on the Extent of Cross-Linking of TnC and TnI[a]

	+calcium, +magnesium	−calcium, +magnesium	−calcium, −magnesium
expt 1a	15[b] (100)[c]	11[b] (73)	7[b] (47)
expt 1b	14 (100)	11 (79)	6 (43)
expt 2	17 (100)	11 (65)	5 (29)
expt 3	30 (100)	21 (70)	12 (40)

[a] In experiments 1a and 1b, the same thin filament preparations were separately irradiated for cross-linking. Different thin filament preparations were used for experiments 1, 2, and 3. In experiments 1 and 2, 0.5 mg/mL TnC was reacted with 1.5 mg/mL ABI for 3 h at 25 °C, while 1.0 mg/mL TnC was reacted with 2.0 mg/mL ABI for 2 h at 25 °C in experiment 3. The fluorescence profile shown in Figure 5 corresponds to experiment 3. [b] The amount (percent) of the fluorescent intensity present in the 32K peak. The total fluorescent intensity on a NaDodSO$_4$ gel is defined as 100%. [c] Numbers in parentheses represent the normalized values of the amount of the fluorescent intensity in the 32K peak. 100 was set for the cross-linking in the presence of calcium and magnesium.

becomes much sharper and more symmetrical. Moreover, the apparent molecular weight of the peak is 32 000.

The assignment of the 32K peak in Figure 5 as the cross-linked product of TnC and TnI is verified by the cross-linking experiment of the thin filament complex containing TnC-(ABI)–TnI(DACM)–TnT in place of TnC(DACM–ABI)–TnI–TnT. After the photo-cross-linking reaction, DACM labels covalently linked to TnI distribute into two peaks. One peak is TnI and the other is the cross-linked product of TnC and TnI. The apparent molecular weight of the latter peak is 32 000, as expected.

The change in the apparent molecular weight of the cross-linked product of TnC and TnI may be due to a change in the site of TnC–TnI contact, since it is very likely that the apparent molecular weights of cross-linked products estimated by NaDodSO$_4$ gel electrophoresis would depend on the position of the cross-links. The presence of a shoulder on the leading edge of the 38K peak can be explained on the basis of the same reasoning as discussed later.

Another interesting feature observed in Figure 5 is that calcium affects the fluorescent intensity of the 32K peak, in contrast to the finding that the fluorescent intensity of the 38K peak in Figure 3 is insensitive to calcium. Moreover, magnesium is also found to affect the fluorescent intensity of the 32K peak. In Figure 5, 30% of the total fluorescent intensity is present in the 32K peak when the cross-linking reaction was carried out in the presence of calcium and magnesium (trace A), whereas 21% is found in the 32K peak in the presence of magnesium but in the absence of calcium (trace B). In the absence of calcium and magnesium, only 12% remains in the peak (trace C). Although the fluorescent intensity of the 56K peak is weak and difficult to quantitate, neither calcium nor magnesium drastically affects the intensity.

Similar experiments were repeated to ensure that the change in the fluorescent intensity of the 32K peak induced by calcium and magnesium is reproducible. Results of three independent experiments are tabulated in Table II. In experiments 1a and 1b, the same thin filament preparations were separately irradiated for cross-linking. In experiments 1 and 2, a lower concentration of ABI (1.5 mg/mL) was used for modifying TnC than in experiment 3 (2 mg/mL); thus, the extent of cross-linking in experiments 1 and 2 was lower than that in experiment 3. However, the extents of calcium- and magnesium-induced changes are similar for these three cases; maximum cross-linking was observed when the reconstituted thin

filament was irradiated in the presence of calcium and magnesium, while the extent of cross-linking of TnC and TnI decreased by 21–35% in the absence of calcium and by 53–71% in the absence of calcium and magnesium.

Discussion

A method has been developed to analyze the arrangement of proteins in complex, multicomponent systems. The use of methyl 4-azidobenzimidate (ABI), a heterobifunctional reagent, makes it possible to embed cross-linkers in a particular component in the system before carrying out the cross-linking reaction by flash photolysis of aryl azides, thus reducing the number of possible cross-linked pairs. Labeling a component in the system with a fluorescent dye also facilitates the assignment of the cross-linked products. By use of the flash photolysis apparatus, the system can be cross-linked in a few milliseconds and the arrangement of proteins in the system is fixed in its "native" state since the cross-linking reaction takes place on a fast time scale compared with that required to unfold proteins (Matheson et al., 1977).

In the present study, the method was applied to investigate the arrangement of troponin components in the thin filament complex in the presence and absence of calcium. The isolated TnC was modified with ABI to embed aryl azides on its surface. The fluorescent dye DACM was also covalently linked to TnC or TnI to make the assignment of cross-linked products easy. These modifications are unlikely to affect the structure of troponin, since troponin reconstituted with a modified component(s) retains its full activity to confer calcium sensitivity to actomyosin ATPase. Thus, using the reconstituted troponin in which TnC carries aryl azides, it becomes possible to study the arrangement of troponin components in situ.

When the troponin reconstituted from TnT, TnI, and the DACM- and ABI-modified TnC was irradiated by a xenon flash with a millisecond duration in 0.1 M KCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) in the presence and absence of calcium, cross-links were formed between TnC and TnI and also between TnC and TnT. Since two functional groups of the cross-linker, an azide and an imido ester, reside within ~5 Å, it is concluded that TnC is in close contact with both TnI and TnT when troponin complex is free in a solution of physiological ionic strength and pH. Although spin-labeling (Ohnishi et al., 1975) and fluorescence-labeling (van Eerd & Kawasaki, 1973) studies have shown calcium-dependent interactions among troponin components even when troponin is free in solution, the present study failed to detect any appreciable calcium-dependent change in the topology of troponin free in solution, consistent with the earlier observation by Hitchcock (1975a) that calcium did not affect the cross-linking of troponin with diimido ester cross-linkers.

Quite surprisingly, it was found that the quaternary structure of troponin changed when it was incorporated in the thin filament complex. The change is easily recognized by comparing fluorescent intensity profiles of $NaDodSO_4$ gels of cross-linked troponin or thin filament complex (Figures 3 and 5). When troponin was incorporated in the thin filament complex, the extent of cross-linking between TnC and TnT was significantly reduced; only a small percent of the total TnC was cross-linked with TnT when troponin was in the thin filament complex, whereas 10% of TnC formed the cross-linked product with TnT for troponin free in solution. The observation indicates that the arrangement of TnC and TnT is different between troponin free in solution and that incorporated in the thin filament complex. Moreover, it was found that the broad fluorescent peak with an apparent molecular weight of 38 000 (seen in Figure 3) changed to a sharp, symmetrical peak with an apparent molecular weight of 32 000 (as shown in Figure 5), suggesting that the arrangement of TnC and TnI is also different between the two cases. The assumption underlying the argument is that the apparent molecular weight of cross-linked polypeptide chains would depend on the position of cross-links. The presence of a shoulder on the leading edge of the 38K peak can be explained by the same reasoning, since it is likely that there are two TnC binding regions in TnI (Syska et al., 1976): one at the N terminal and the other in the middle of the polypeptide chain, thus producing two species of cross-linked product.

The quaternary structure of troponin in the thin filament complex is calcium sensitive, and the cross-linking of TnC and TnI is affected by calcium, in contrast to the finding that no appreciable change is induced by calcium in the cross-linking pattern of troponin when it is free in solution. As shown in Table II, maximum cross-linking of TnC and TnI was detected when the thin filament complex was photo-cross-linked in the presence of calcium and magnesium. In the absence of calcium, however, the extent of TnC–TnI cross-linking decreased by 21–35%. Magnesium was also found to affect the TnC–TnI cross-linking in a similar way as calcium; when the thin filament complex was cross-linked in the absence of calcium and magensium, the extent of the TnC–TnI cross-linking decreased by 53–71% compared to the maximum cross-linking. On the basis of gel electrophoresis of TnC–TnI complex (Perry et al., 1972; van Eerd & Kawasaki, 1973) or other physicochemical methods (van Eerd & Kawasaki, 1973; Ohnishi et al., 1975), it has been previously assumed that the contact between TnC and TnI is sensitive to calcium. However, the present study is the first to show that the TnC–TnI contact is really dependent on calcium and magnesium in situ.

When the thin filament complex was photo-cross-linked, it was also observed that the cross-linking of TnC and TnT remained at a low level irrespective of the concentration of calcium and magnesium, suggesting that no drastic change was induced in the TnC–TnT contact by the divalent cations.

In the present study, interactions of TnC with TnI and TnT in the reconstituted troponin or thin filament complex were investigated by the millisecond photo-cross-linking method. Other pairs of interactions such as actin–TnI and TnI–TnT are now under study by using reconstituted troponins containing the DACM- and ABI-modified TnI or TnT. These studies, together with the present investigation, will reveal the architecture of the organization of proteins in the thin filament complex and thus the molecular mechanism of relaxation and activation of muscle triggered by calcium.

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Synthesis and Characterization of Phosphocitric Acid, a Potent Inhibitor of Hydroxylapatite Crystal Growth†

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ABSTRACT: Human urine and extracts of rat liver mitochondria contain apparently identical agents capable of inhibiting the precipitation or crystallization of calcium phosphate. Its general properties, as well as ^{1}H NMR and mass spectra, have suggested that the agent is phosphocitric acid. This paper reports the synthesis of phosphocitric acid via the phosphorylation of triethyl citrate with *o*-phenylene phosphochloridate, hydrogenolysis of the product to yield triethyl phosphocitrate, hydrolytic removal of the blocking ethyl groups, and also chromatographic purification. An enzymatic assay of phosphocitrate is described. Synthetic phosphocitrate was found to be an exceedingly potent inhibitor of the growth of hydroxylapatite seed crystals in a medium supersaturated with respect to Ca^{2+} and phosphate. Comparative assays showed phosphocitrate to be much more potent than the most active precipitation–crystallization inhibitors previously reported, which include pyrophosphate and ATP. ^{14}C-Labeled phosphocitrate was bound very tightly to hydroxylapatite crystals. Such binding appeared to be essential for its inhibitory activity on crystal growth. Citrate added before, but not after, phosphocitrate greatly enhanced the inhibitory potency of the latter. This enhancement effect was not given by other tricarboxylic acids. The monoethyl ester of phosphocitrate had no inhibitory effect on hydroxylapatite crystal growth.

Biochemical agents capable of inhibiting the precipitation and/or crystallization of calcium phosphate have been detected in urine (Howard & Thomas, 1958; Howard, 1976; Fleisch & Bisaz, 1962), saliva (Gron & Hay, 1976), bile (Sutor & Percival, 1976), and other body fluids. Such inhibitory agents have also been detected (Tew & Mahle, 1977) in extracts of rat liver mitochondria, which can accumulate large amounts of Ca^{2+} and phosphate during respiration to form nondiffracting electron-dense deposits of "amorphous" calcium phosphate (Greenawalt et al., 1964). Similar inhibitors have also been detected in extracts of the hepatopancreas of the blue crab, *Callinectes sapidus*, in which large amounts of calcium phosphate are stored during ecdysis, also in a nondiffracting

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