Millisecond Photo-Cross-Linking of Protein Components in Vertebrate Striated Muscle Thin Filaments†

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ABSTRACT: Troponin I (TnI) was reacted with a photosensitive heterobifunctional reagent, methyl 4-azidobenzimidate (ABI), and then troponin was reconstituted with the ABI-modified TnI. Flash irradiation of the reconstituted troponin resulted in the formation of cross-links between TnI and other components of troponin, troponin C (TnC) and troponin T (TnT), suggesting that TnI is in contact with TnC and TnT when troponin is free in solution. No effect of calcium on the cross-linking could be detected. When the reconstituted troponin was complexed with F-actin–tropomyosin, flash irradiation of the reconstituted thin filament yielded the cross-linked products of TnC–TnI, TnI–TnT, and TnI–actin in the presence and absence of calcium, indicating that TnI is in contact with TnC, TnT, and actin in the thin filament complex irrespective of calcium concentration. No cross-linking could be detected between TnI and tropomyosin. Calcium was found to affect the cross-linking of TnC–TnI and TnI–actin; when TnC was saturated with calcium, the extent of the TnC–TnI cross-linking increased, while that of the TnI–actin cross-linking decreased. Calcium did not affect the TnI–TnT cross-linking.

Millisecond photo-cross-linking using an aryl azide cross-linker has been proved to be a promising method to investigate the arrangement of protein components in complex, multicomponent systems (Sutoh, 1980). The technique exploits the following properties of the aryl azide group (Bayley & Knowles, 1977). The aryl azide is chemically inert unless it is irradiated at a wavelength less than 350 nm. The irradiation of the aryl azide yields an extremely reactive species, nitrene, whose lifetime is very short (<1 ms) in an aqueous solution (Reiser et al., 1971). Thus, when flash photolysis apparatus is used, we find that photo-cross-linking can fix a protein complex in its "native" state before gross structural changes, if any, are induced by cross-linking reactions, since the time scale needed for completion of the cross-linking (a few milliseconds) is shorter than that required for gross structural changes in a protein (Matheson et al., 1977).

In a previous paper (Sutoh, 1980), the millisecond photo-cross-linking of the reconstituted troponin or thin filament containing troponin C (TnC),[1] which carried aryl azide groups, has revealed interesting interactions of TnC with other protein components. When the reconstituted free troponin, in a solution of physiological ionic strength and pH, was irradiated with a xenon flash to form cross-links, TnC was found to be cross-linked with two other components of troponin, troponin I (TnI) and troponin T (TnT). The effect of calcium on the TnC–TnI and TnC–TnT cross-linkings could not be detected. Once the reconstituted troponin was incorporated in the thin filament complex, the arrangement of the troponin components was found to be appreciably different from that free in solution, judging from differences in the cross-linking pattern between the two cases. Furthermore, it was observed that the TnC–TnI cross-linking was sensitive to calcium and magnesium when the toponin was in the thin filament complex, suggesting that the TnC–TnI contact in situ is calcium and magnesium dependent. The TnC–TnT cross-linking remained insignificant irrespective of calcium or magnesium concentration when the thin filament complex was photo-cross-linked.

In the present work, we investigated interactions of TnI with other components of the thin filament complex, that is, actin, tropomyosin, TnT, and TnC, employing the same method described above. Photo-cross-linking of the thin filament complex containing TnI modified with a photosensitive heterobifunctional reagent, methyl 4-azidobenzimidate (ABI), resulted in cross-linkings of TnI with actin, TnT, and TnC, indicating that TnI is in contact with these components. No cross-linking was detected between TnI and tropomyosin. Calcium was found to affect the TnC–TnI cross-linking and also the TnI–actin cross-linking, suggesting the calcium-dependent change in the quaternary structure of the thin filament complex.

Materials and Methods

Preparation of Proteins. Troponin and its components TnC and TnT were prepared according to Ebashi et al. (1971) and Ebashi (1974), and TnI was prepared using the method of Sutoh (1980). They were homogeneous when characterized by $NaDodSO_4$ gel electrophoresis. Desensitized actomyosin was prepared according to Syska et al. (1976). Actin content in the preparations was about 25% (weight), judging from the intensity of coomassie blue staining of $NaDodSO_4$ gels. Neither troponin nor tropomyosin was detected on $NaDodSO_4$ gels of the preparations. Actin was prepared according to Spudich & Watt (1971), and tropomyosin was prepared according to Wakabayashi et al. (1975).

Modifications of TnI with DACM and ABI. Whole troponin (5 mg/mL) in 5 mM imidazole (pH 7.0) was labeled with 1.2 molar equivalent of *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) (Machida et al., 1975) for 1 h at 0 °C. The reaction was terminated by the addition of 2 mM (final concentration) *N*-acetylcysteine. Under these conditions, only TnI was labeled with the fluorescent dye. The labeled TnI was isolated and purified following the method described previously (Sutoh, 1980). The DACM-labeled TnI was finally eluted from SP-Sephadex

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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; Dns, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

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C-50 with 6 M urea, 0.1 M NaCl, 4 mM mercaptoethanol, 1 mM EDTA, and 2 mM $NaHCO_3$ (pH 8.0), and stored in the solvent at −20 °C.

The DACM-labeled TnI (0.6 mg/mL) in the above solvent was mixed with a third volume of ABI (3 mg/mL) in 0.4 M $NaHCO_3$–Na_2CO_3 (pH 9.4). The amidination reaction was allowed to proceed for 2 h at 25 °C and then terminated by the addition of 0.02 volume of 1 M lysine; the resulting solution was dialyzed against 6 M urea, 10 mM imidazole, and 1 mM 2-mercaptoethanol (pH 7.0). The DACM- and ABI-modified TnI was denoted as TnI(DACM–ABI). When TnI without DACM labeling was used for the ABI modification, the same procedure was employed as described above. The ABI-modified TnI was denoted as TnI(ABI). All procedures after the ABI modification were carried out in the dark or under a red safety light.

DACM Labeling of Actin and Dns Labeling of Tropomyosin and TnT. F-actin (4.4 mg/mL) in 20 mM imidazole (pH 7.0) was reacted with a threefold molar excess of DACM for 10 min at 4 °C, and the reaction was terminated by the addition of 2 mM (final concentration) *N*-acetylcysteine. The DACM-modified actin was denoted as actin(DACM).

Modification of tropomyosin with DACM resulted in inefficient labeling; therefore, dansyl chloride (Dns) was used for the fluorescence labeling of tropomyosin. Tropomyosin (2 mg/mL) in 0.5 M NaCl and 10 mM $NaHCO_3$ (pH 9.0) was treated with a 10-fold molar excess of Dns for 20 h at 4 °C. The reaction was terminated by the addition of 10 mM (final concentration) lysine.

TnT (1.7 mg/mL) in 0.5 M NaCl and 10 mM $NaHCO_3$ (pH 9.0) was reacted with a fivefold molar excess of Dns for 9 h at 4 °C, and the reaction was terminated by the addition of lysine as described above. The Dns-modified TnT was denoted as TnT(Dns).

Reconstitution of Troponin and the Thin Filament Complex. Troponin was reconstituted by placing equal molar amounts of TnC, TnI, and TnT in 6 M urea and then dialyzing the mixture successively against 0.5, 0.25, and 0.1 M NaCl containing 10 mM imidazole (pH 7.0). The resulting clear solution was centrifuged at 100000*g* for 2 h, and the supernatant was used as the reconstituted troponin.

The thin filament complex was reconstituted by mixing F-actin in 0.1 M NaCl and 10 mM imidazole (pH 7.0) and tropomyosin in the same buffer in a 7:1 molar ratio. The resulting F-actin–tropomyosin complex was then mixed with the reconstituted troponin in such a way that the molar ratio of actin, tropomyosin, and troponin in the mixture was 7:1:1. When TnI modified with ABI was used for reconstitution, all procedures were carried out in the dark or under a red safety light.

Measurement of Calcium Sensitivity. The ATPase activity of a mixture of desensitized actomyosin (0.13 mg/mL), tropomyosin (10 μg/mL), and troponin (9 μg/mL) in 50 mM KCl, 10 mM imidazole, and 2 mM $MgCl_2$ (pH 7.0) was measured in the presence of 0.1 mM $CaCl_2$ or 0.5 mM EGTA. In the reaction mixture, the molar ratio of actin, tropomyosin, and troponin was 7:1.3:1, using the above estimate that about 25% (weight) of the total protein in the desensitized actomyosin preparations was actin. ATP hydrolysis was initiated by the addition of ATP to the mixture, allowed to proceed for 5 min at 25 °C, and then terminated by the addition of an equal volume of 20% trichloroacetic acid. Phosphate liberation was determined by the method of Fiske & SubbaRow (1925). Calcium sensitivity was determined as [1 − (EGTA ATPase/Ca^{2+} ATPase)] × 100 (%), where EGTA ATPase stands for the ATPase activity in the presence of EGTA and Ca^{2+} ATPase is that in the presence of calcium.

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

	0.1 mM $CaCl_2$	0.5 mM EGTA	Ca sensitivity (%)[b]
no troponin	0.370[c]	0.363	2
native troponin	0.370	0.136	63
TnT–TnI–TnC	0.382	0.127	67
TnT–TnI(DACM)–TnC	0.370	0.129	65
TnT–TnI(ABI)–TnC	0.354	0.101	71
TnT–TnI(DACM-ABI)–TnC	0.370	0.129	65

[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 0.13 mg of desensitized actomyosin and 10 μg of tropomyosin in 1 mL. When troponin was used, 9 μg was added to the mixture. The ATPase reaction was initiated by the addition of 0.1 mL of 10 mM ATP, allowed to proceed for 5 min, and then terminated by the addition of 1 mL of 20% trichloroacetic acid. [b] Calcium sensitivity = [1 − (EGTA ATPase/Ca^{2+} ATPase)] × 100 (%). [c] Micromoles of phosphate per milligram of actomyosin per minute.

Miscellaneous Procedures. The reconstituted troponin or thin filament complex was photo-cross-linked with flash irradiation using a xenon flash tube as described previously (Sutoh, 1980).

$NaDodSO_4$ gel electrophoresis was carried out according to Laemmli (1970), using 10% acrylamide–0.3% bis(acrylamide) for the separation gel. The $NaDodSO_4$ gels were scanned by a fluorescence gel scanner immediately after electrophoresis, and the fluorescent intensity of each peak was quantitated as described previously (Sutoh, 1980).

The amount of free amino groups in TnI after the amidination reaction with ABI was determined by the method of Habeeb (1966). Of the total amino groups in TnI, 10% was found to be modified with ABI under the conditions described above.

Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Chemicals. All chemicals were reagent grade. ABI was synthesized according to Ji (1977) and Lewis et al. (1977), and its purity was checked by thin-layer chromatography and infrared spectroscopy.

Results

Effect of the DACM and ABI Modifications of TnI on Its Function. Before we carried out the photo-cross-linking experiments using the DACM- and ABI-modified TnI, the effects of these modifications on the function of TnI were investigated by measuring the ability of reconstituted troponins to confer calcium sensitivity on the actomyosin ATPase. The ATPase activity of a mixture of desensitized actomyosin (Syska et al., 1976), tropomyosin, and troponin reconstituted with the DACM- and/or ABI-modified TnI was measured in the presence and absence of calcium as described under Materials and Methods. As a control, troponin, which has never experienced any dissociation or modification procedures (native troponin), was used in place of the reconstituted troponins. Typical results are shown in Table I. The calcium sensitivity of native troponin was calculated to be 63%, while that of troponin reconstituted from TnT, TnC, and TnI without any modification was 67%. When TnI(DACM), TnI(ABI), or TnI(DACM–ABI) was used for reconstituting the troponin complex, the calcium sensitivity was 65, 71, or 65%, respectively. The experimental error in calculating the calcium

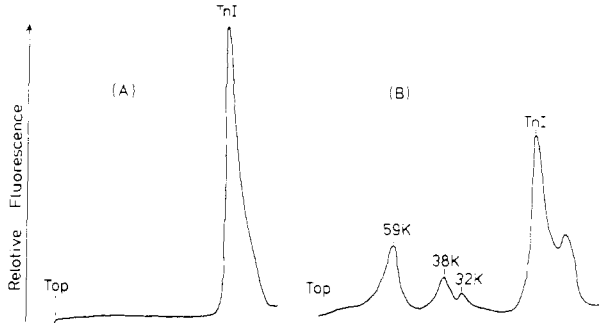

FIGURE 1: Cross-linking of the reconstituted troponin, TnT–TnI-(DACM–ABI)–TnC. Traces of fluorescence scanning of $NaDodSO_4$ gels of the reconstituted troponin complex before (A) and after (B) irradiation with xenon flash. The fluorescent intensity of DACM covalently linked to TnI was scanned by a fluorescence gel scanner. Conditions for the photo-cross-linking are: solvent, 0.1 M NaCl, 10 mM imidazole, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (pH 7.0); temperature, 25 °C.

sensitivity was about 10%. The results lead us to conclude that neither DACM nor ABI modification damages the function of TnI.

Cross-Linking of the Reconstituted Troponin. Troponin reconstituted from TnT, TnC, and TnI(DACM–ABI) was irradiated with a xenon flash in 0.1 M NaCl, 10 mM imidazole, and 1 mM $MgCl_2$ (pH 7.0) in the presence of 0.1 mM $CaCl_2$ or 0.5 mM EGTA, to form cross-links between TnI and other troponin components in contact with TnI. The cross-linked products were analyzed by $NaDodSO_4$ gel electrophoresis. $NaDodSO_4$ gels were scanned by a fluorescence gel scanner, and distribution of fluorescent intensity of DACM covalently linked to TnI was quantitated along the gels.

Figure 1 shows traces of fluorescence gel scanning of $NaDodSO_4$ gels of the reconstituted troponin, TnT–TnI (DACM–ABI)–TnC, before (A) and after (B) irradiation with a xenon flash. Before irradiation, only the TnI peak was observed on the gel, while three fluorescent peaks corresponding to the cross-linked products were detected on the gel after irradiation. In addition, the TnI peak was found to split into two, one major and one minor, after irradiation, as observed in Figure 1. It must be noted that no appreciable cross-linking was observed when the troponin complex without aryl azide groups was irradiated with a xenon flash.

The apparent molecular weights of the cross-linked products estimated from their mobilities on $NaDodSO_4$ gels were 59 000, 38 000, and 32 000, respectively. Based on the cross-linking experiments of the reconstituted troponin, TnT–TnI–TnC(DACM–ABI), or TnT–TnI(DACM)–TnC(ABI), it has been previously reported (Sutoh, 1980) that the 38K and 32K peaks correspond to the cross-linked product of TnC and TnI. The 59K peak was identified as the cross-linked product of TnI-TnT, since a fluorescent peak with an apparent molecular weight of 59 000 was again observed on $NaDodSO_4$ gels, when the photo-cross-linking was carried out on the reconstituted troponin in which a fluorescent dye (Dns) was covalently linked to TnT in place of TnI and aryl azide groups were embedded on TnI as before. The minor peak that appeared on the shoulder of the major TnI peak after irradiation possibly was the result of internal cross-linking of TnI.

Quantitative analysis of the fluorescence profiles revealed that 15% of the total fluorescence observed on a gel was found in the 59K peak, while the 38K and 32K peaks contained 10 and 4% of the total fluorescent intensity, respectively. No appreciable calcium-dependent difference was detected in the fluorescence profiles, consistent with the earlier observation (Sutoh, 1980) that calcium did not affect the cross-linking

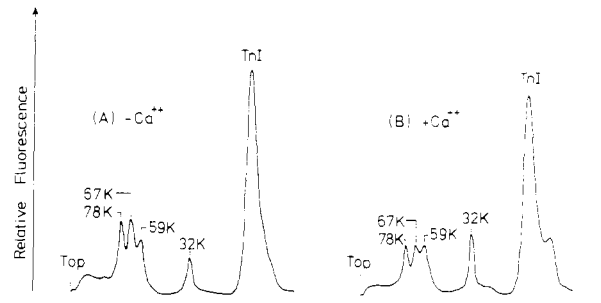

FIGURE 2: Cross-linking of the reconstituted thin filament complex containing TnT–TnI(DACM–ABI)–TnC. Traces of fluorescence scanning of $NaDodSO_4$ gels of the thin filament preparations photo-cross-linked in (A) 0.1 M NaCl, 10 mM imidazole, 1 mM $MgCl_2$, and 0.5 mM EGTA (pH 7.0), or in (B) 0.1 M NaCl, 10 mM imidazole, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (pH 7.0). The thin filament preparations were irradiated at 25 °C.

pattern of the reconstituted troponin, TnT–TnI–TnC-(DACM–ABI).

Cross-Linking of the Reconstituted Thin Filament Complex. The reconstituted troponin, TnT–TnI(DACM–ABI)–TnC, was complexed with F-actin–tropomyosin, and the resulting thin filament complex was irradiated with a xenon flash in 0.1 M NaCl, 10 mM imidazole, and 1 mM $MgCl_2$ (pH 7.0) in the presence of 0.1 mM $CaCl_2$ or 0.5 mM EGTA. Figure 2 shows traces of fluorescence scanning along $NaDodSO_4$ gels of reconstituted thin filament preparations cross-linked in the absence of calcium (A) and in its presence (B). The photo-cross-linking reaction of the thin filament complex yielded four cross-linked species with apparent molecular weights of 78 000, 67 000, 59 000, and 32 000. No appreciable cross-linking was detected when the thin filament complex without aryl azide groups was irradiated with a xenon flash.

The 32K peak has already been identified as the cross-linked product of TnC and TnI (Sutoh, 1980). The 59K peak was identified as the cross-linked product of TnI and TnT on the basis of an experiment similar to that mentioned above; photo-cross-linking of the thin filament complex containing the reconstituted troponin, TnT(Dns)–TnI(ABI)–TnC, yielded a fluorescent peak with an apparent molecular weight of 59 000 on $NaDodSO_4$ gels.

When actin was labeled with the fluorescent dye DACM and aryl azide groups were embedded in TnI, irradiation of the reconstituted thin filament complex with a xenon flash yielded fluorescent species with apparent molecular weights of 67 000 and 78 000. Furthermore, fluorescent intensity of these peaks were sensitive to calcium in a similar way, as observed for the 67K and 78K peaks in Figure 2 (shown later). Thus, it was concluded that the 67K and 78K species were the cross-linked products of actin and TnI. Note that the cross-linked products of TnC and TnI also appeared as double peaks (32K and 38K) on $NaDodSO_4$ gels when troponin free in solution was photo-cross-linked (Figure 1).

Since several groups (Potter & Gergely, 1974; Hitchcock, 1975a; Maruyama et al., 1975) have reported the possibility of interaction between TnI and tropomyosin, photo-cross-linking was carried out on the thin filament complex containing tropomyosin labeled with Dns and TnI modified with ABI, to see if the cross-linked product of TnI and tropomyosin was not detected because it comigrated with other cross-linked species on $NaDodSO_4$ gels. No fluorescent peak corresponding to the cross-linked product of TnI and tropomyosin was detected, showing that TnI was not cross-linked to tropomyosin under the present experimental conditions.

Effect of Calcium on the Quaternary Structure of the Thin Filament. It has been shown that TnC–TnI cross-linking is

Table II: Effect of Calcium on the Extent of Cross-Linking[c]

cross-linked species	app mol wt	fluorescent intensity (%)[a] −calcium	fluorescent intensity (%)[a] +calcium	−calcium/ +calcium
		3.4	6.9	0.57
TnC-TnI	32 000	4.8	6.1	0.79
		5.4	7.2	0.75
		11	14	0.79
TnC-TnI[b]	32 000	11	17	0.65
		21	30	0.70
		6.8	7.4	0.92
TnI-TnT	59 000	5.5	6.0	0.92
		8.7	9.4	0.93
		9.7	7.1	1.4
TnI-actin	67 000	8.9	6.7	1.3
		9.6	6.2	1.5
		8.9	5.6	1.6
TnI-actin	78 000	7.2	4.9	1.6
		9.1	6.9	1.3

[a] $NaDodSO_4$ gels were scanned by a fluorescence gel scanner and the fluorescent intensity of each peak was quantitated. Fluorescent intensity (%) = (fluorescent intensity of a peak/total fluorescent intensity of a gel) × 100. [b] Taken from our previous paper (Sutoh, 1980). The photo-cross-linking reaction was carried out on the reconstituted thin filament complex containing the DACM- and ABI-modified TnC (TnC(DACM-ABI)). [c] Photo-cross-linking reactions were carried out in 0.1 M NaCl, 10 mM imidazole, and 1 mM $MgCl_2$ (pH 7.0) in the presence of 0.5 mM EGTA (−calcium) or 0.1 mM $CaCl_2$ (+calcium).

calcium dependent when the thin filament complex was photo-cross-linked (Sutoh, 1980). In the present experiment, the effect of calcium on the cross-linking of the thin filament complex was observed to be similar. Table II shows the results of quantitation of the amount of fluorescent intensity present in each peak seen on $NaDodSO_4$ gels in Figure 2. Three independent experiments were tabulated in the table. It is easily recognized that TnI–TnT cross-linking remains at a similar level irrespective of the calcium concentration; in contrast, the level of TnC–TnI or TnI–actin cross-linking depends on calcium. The ratio of the amount of fluorescent intensity present in each peak in the absence of calcium to that in its presence (−calcium/+calcium) is 0.57–0.79 for the TnC–TnI cross-linking and 1.3–1.5 (for the 67K peak) or 1.3–1.6 (for the 78K peak) for the TnI–actin cross-linking. In other words, the extent of the TnC–TnI cross-linking is reduced by 21–43% in the absence of calcium, while that of the TnI–actin cross-linking is increased by 30–60%.

It should be noted that virtually the same amount of calcium-induced change was observed in TnC–TnI cross-linking when ABI-modified TnC was used in place of ABI-modified TnI, as shown in Table II. For the thin filament complex containing the ABI-modified TnC, the (−calcium/+calcium) ratio is 0.65–0.79 (Sutoh, 1980), while it is 0.57–0.79 for the thin filament complex containing the ABI-modified TnI.

Another calcium-dependent difference was noticed in the fluorescence profiles in Figure 2. When the thin filament complex was irradiated in the presence of calcium (trace B), appreciable amounts of fluorescent materials appeared on the leading edge of the TnI peak; however, no shoulder was observed on the edge of the TnI peak for the thin filament complex cross-linked in the absence of calcium (trace A). The observation implies that calcium affects the tertiary structure of TnI through the TnC–TnI interaction and consequently the extent of its internal cross-linking.

Discussion

In order to understand the molecular mechanism of troponin-controlled regulation of the contraction–relaxation cycle of muscle, one has to understand the mode of association of five major components of the thin filament complex (actin, tropomyosin, TnT, TnI, and TnC) in the presence and absence of calcium. Although various chemical and physicochemical techniques have been applied to detect interactions among these components (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), no work has yet been done to study the interactions in the presence of all thin filament components necessary for the calcium regulation of contraction.

In our previous study (Sutoh, 1980), the millisecond photo-cross-linking method had been successfully applied to investigate the quaternary structure of troponin in the thin filament complex. By monitoring cross-linking between TnC and TnI and that between TnC and TnT, we found that the quaternary structure of troponin in the thin filament is different from that free in solution, and that the former is dependent on calcium.

In the present paper, the same technique was employed to study interactions of TnI with other components of troponin or the thin filament complex. When the reconstituted troponin containing TnI that carried aryl azide groups (TnI(DACM–ABI)) was photo-cross-linked in the presence or absence of calcium, appreciable amounts of cross-linking between TnI and TnT and between TnI and TnC were detected, as shown in Figure 1, suggesting that TnI is in contact with TnC and TnT when troponin is free in solution. Calcium-induced changes in the cross-linking could not be detected, consistent with the previous reports (Hitchcock, 1975a; Sutoh, 1980). Because of the discovery that TnC is in contact with TnI and TnT when troponin is free in solution (Sutoh, 1980), it is concluded that three components of troponin free in solution are in mutual contact and their arrangement is rather insensitive to calcium.

When reconstituted troponin was complexed with F-actin–tropomyosin, photo-cross-linking of the reconstituted thin filament complex produced cross-linking patterns (Figure 2) different from that resulting from photo-cross-linking of the reconstituted troponin alone (Figure 1). The cross-linked products of TnC and TnI observed as the double peaks (32K and 38K) in Figure 1 appeared as a single peak (32K) in Figure 2, indicating a change in the arrangement of TnC and TnI in the troponin complex when troponin is incorporated in the thin filament. Exactly the same phenomenon was observed when the ABI-modified TnC was used in place of the ABI-modified TnI (Sutoh, 1980).

Another difference between the two cross-linking profiles is that two peaks (67K and 78K) appeared above the cross-linked product of TnI and TnT (the 56K peak) on $NaDodSO_4$ gels when the thin filament complex was photo-cross-linked. They were identified as the cross-linked products of TnI and actin, on the basis of the photo-cross-linking experiment of the reconstituted thin filament containing the DACM-modified actin and the ABI-modified TnI. Cross-linking between TnI and tropomyosin could not be detected. These observations indicate that TnI is in contact with actin, TnT, and TnC in the thin filament.

As easily recognized in Table II, calcium affects the arrangement of these components in the thin filament complex; the extent of cross-linking of TnC–TnI and that of TnI–actin depends on the calcium concentration, whereas that of TnI–TnT is virtually independent of calcium. The ratio of the

amount of cross-linked materials produced in the absence of calcium to that produced in its presence (−calcium/+calcium in Table II) is 0.57–0.79 for the TnC–TnI cross-linking and 1.3–1.5 (for the 67K peak) or 1.3–1.6 (for the 78K peak) for the TnI–actin cross-linking. Thus, the effect of calcium is just the reverse for TnC–TnI cross-linking and for TnI–actin cross-linking.

The calcium-induced increase (or decrease) of the extent of cross-linking mentioned above may result from an increase (or decrease) of contact surface area between the two components participating in the cross-linking. The other possibility is that the two components move around each other, retaining the same extent of contact surface area between them. The latter possibility must be taken into account, since aryl azides are not necessarily distributed uniformly on a protein surface, even though nitrenes generated from aryl azides can react with various bonds in their proximity in nonspecific fashion (Bayley & Knowles, 1977). However, the finding that the extent of calcium-induced change in the TnC–TnI cross-linking is virtually the same for the thin filament containing the ABI-modified TnC and for that containing the ABI-modified TnI seems to favor the former possibility at least for the TnC–TnI cross-linking. In other words, the contact between TnC and TnI in the thin filament seems to be strengthened on saturating TnC with calcium. The conclusion is in agreement with the previous observation, based on urea gel electrophoresis of the TnC–TnI complex, that the TnC–TnI complex dissociated into two components in the absence of calcium, while its integrity was preserved in the presence of calcium (Perry et al., 1972).

As for TnI–actin cross-linking, it is somewhat surprising that TnI is cross-linked with actin even in the presence of calcium, since it is generally assumed that TnI dissociates from actin in the presence of calcium (Hitchcock et al., 1973; Potter & Gergely, 1974; Hitchcock, 1975b). Although the extent of TnI–actin cross-linking is reduced in the presence of calcium (Table II), the cross-linked products of TnI and actin (the 67K and 78K species) are still the major cross-linked species observed on $NaDodSO_4$ gels. Considering the fact that the photo-cross-linking reaction was completed within a few milliseconds, it is unlikely that the cross-linking resulted from the thermal collision of the two components. Thus, it is concluded that TnI is in contact with actin even in the presence of calcium, though the mode of their contact in the presence of calcium is different from that in its absence.

The finding that two peaks were observed for the cross-linked products of TnC and TnI (the 32K and 38K peaks in Figure 1) and for those of TnI and actin (the 67K and 78K peaks in Figure 2) requires explanation. As for the cross-linked products of TnC and TnI, it is likely that the 32K and 38K peaks contain cross-linked polypeptide chains with different configurations, since TnI probably contacts with TnC at two different sites in its primary sequence, one at the N-terminal and the other in the middle of the peptide chain (Syska et al., 1976). The mobility of cross-linked polypeptide chains in the $NaDodSO_4$ gel would depend on the site(s) of crosslink(s) even when they have the same molecular weight. The same reasoning may be applied to the cross-linked products of TnI and actin, although further experimentation is needed to prove it.

Although there are some reports showing that TnI is in contact with TnT (Hitchcock, 1975a; Horwitz et al., 1979), the present work is the first to present convincing evidence that TnI is in contact with TnT in the thin filament complex in the presence and absence of calcium. As noted above, calcium does not affect the extent of the TnI–TnT cross-linking.

In summary, the millisecond cross-linking method has revealed that TnI is in contact with TnC, TnT, and actin in the thin filament irrespective of calcium concentration. It has also shown the calcium-dependent changes in the quaternary structure of the thin filament complex; on saturating TnC with calcium, the extent of TnC–TnI cross-linking increases, while that of the TnI–actin cross-linking is reduced. The TnI–TnT cross-linking is not affected by calcium.

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