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Cross-Linking of Troponin with Dimethylimido Esters†

Sarah E. Hitchcock

ABSTRACT: The topology of troponin, the calcium binding regulatory protein in muscle, has been studied by cross-linking with different length dimethylimido esters. The results show that the three components of troponin are close to each other and that the troponin-I and -T are preferentially cross-linked being 0.6 nm or less apart. The largest cross-linked product is a complex which corresponds in molecular weight to the native troponin complex of 1 mol of each of the three components. Cross-linked troponin has lost the ability to make the actomyosin ATPase calcium sensitive although it does bind to actin-tropomyosin and tropomyosin, and it binds calcium normally. No effect of calcium on cross-linking could be detected.

Muscle contraction is regulated by the free calcium ion concentration (Heilbrun and Wiercinski, 1947). At low calcium concentrations (less than 10^{-8} *M*), the muscle is relaxed and the myosin cross-bridges on the thick filaments cannot interact with the actin containing thin filaments. At higher calcium ion concentrations (10^{-6} *M* or greater), actin-myosin interaction is allowed and contraction proceeds (Huxley, 1969). In vertebrate striated muscle, troponin, associated with actin and tropomyosin in the thin filaments, binds calcium and regulates contraction (Ebashi and Endo, 1968).

Troponin consists of three different proteins: troponin-T (37000 molecular weight in rabbit) which binds to tropomyosin, troponin-I (24000) which inhibits actin-myosin interaction in the presence and absence of calcium, and troponin-C (17800) which binds calcium (cf. Greaser and Gergely, 1971). The functional troponin which confers calcium-sensitive regulation on the actomyosin ATPase is composed of 1 mol of each of these components (Hartshorne and Driezen, 1973; Potter, 1974). The interactions among these proteins have been studied in many laboratories using a variety of chemical, physical, and structural techniques (see review by Weber and Murray, 1973).

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Troponin-I–troponin-C and troponin-T–troponin-C interactions are extensively documented (cf. Wakabayashi and Ebashi, 1968; Ebashi et al., 1973; Greaser and Gergely, 1973; Margossian and Cohen, 1973; Perry et al., 1973; van Eerd and Kawasaki, 1973). However, it has been difficult to demonstrate interaction between troponin-I and -T since they are both highly charged basic proteins which are rather insoluble at physiological ionic strength. Although troponin-T and troponin-I copurify in some conditions and cosediment in the analytical ultracentrifuge (Hartshorne et al., 1969; Schaub and Perry, 1969), and interact with tropomyosin to give a special form of paracrystal (Yamaguchi et al., 1974), this is not evidence for specific complex formation. In the absence of crystallographic data, the arrangement of components within troponin and the shape of the complex are not known in any detail.

Bifunctional imido esters have been used to study the subunit structure of multicomponent proteins (e.g., Davies and Stark, 1970), chromatin (Kornberg and Thomas, 1974), and the surface organization of larger structures such as ribosomes (Slobin, 1972). The dimethylimido esters covalently cross-link lysine ϵ-amino groups of proteins without altering the charge of the protein and the products can be characterized by dodecyl sulfate polyacrylamide gel electrophoresis. In this study dimethylimido esters have been used to cross-link troponin to learn more about the topological arrangement of the subunits: which components are closest to each other and how close?

The use of cross-linkers to study their arrangement has resulted in the important new finding that troponin-T and -I lie close to each other, 0.6 nm or less. From these cross-linking experiments, and the knowledge that these components both interact with troponin-C, it seems likely that all three components are close to each other assuming a compact rather than elongated shape. Although calcium is known to influence interaction among these components, no detectable effect on cross-linking has been observed. The effect of cross-linking on the function of troponin has also been investigated.

Materials and Methods

Preparation of Proteins. Troponin was prepared from the back and thigh muscles of New Zealand rabbits according to Greaser and Gergely (1971) with the modification that 10^{-4} *M* phenylmethanesulfonyl fluoride was added to the initial washes and 1.0 m*M* EDTA was added to the extraction medium.[1] This seemed to reduce proteolysis. Troponin was further purified on SP Sephadex C-50 (Schaub and Perry, 1969) and was stored frozen at −20°C. Tropomyosin was prepared from chicken breast muscle according to Bailey (1948) as modified by Lehman and Szent-Gyorgyi (1972). It was stored frozen at −20°C. Rabbit acetone powder for actin was prepared according to Straub (1942), purified using the procedure of Mommaerts (1952), and stored at −20°C. Actin was extracted from the acetone powder at 0°C with 5 m*M* Tris-HCl (pH 8.0) and 0.01 m*M* ATP and polymerized once at room temperature with 10 m*M* NaCl–0.5 m*M* $MgCl_2$ (Drabikowski and Gergely, 1962). Rabbit myosin was prepared according to Szent-Gyorgyi (1951) as modified by Mommaerts and Parrish (1951) and stored in 50% glycerol, 50% 0.6 *M* NaCl, and 5 m*M* phosphate buffer (pH 7.0) at −20°C. Before use, myosin was precipitated in 0.05 *M* NaCl–5 m*M* phosphate buffer (pH 6.2). The precipitate was collected by centrifugation, dissolved in 0.6 *M* NaCl–5 m*M* imidazole (pH 7.0), and clarified by centrifugation.

Preparation of Imido Esters. Dimethyl suberimidate was prepared from suberonitrile (Aldrich) according to Davies and Stark (1970). The melting point (uncorrected) of the product was 219–225°C (literature value 222–224°C, Slobin, 1972). Methyl acetimidate was synthesized from acetonitrile (Aldrich) as described by Hunter and Ludwig (1972). The melting point of the product was 88–92°C (literature value 93–95°C; Hunter and Ludwig, 1972). These reagents were stored dried in sealed vials (not in vacuo) at −20°C. After opening, the reagent was stored dry, in vacuo, at −20°C. The other dimethylimido esters (see Figure 2) were the generous gift of Dr. John Coggins and were synthesized from their respective nitriles by the same method as the dimethyl suberimidate. Their purity had been checked by nuclear magnetic resonance. These reagents were stored dry, in vacuo, at −20°C.

Amidination of Proteins. Troponin was diluted to 1 mg/ml and dialyzed against 0.1 *M* NaCl, 0.1 *M* sodium borate (pH 8.0) or 0.1 *M* triethanolamine (pH 8.0), 2 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol (Calbiochem) and sedimented for 4–5 hr at 100000*g* to remove aggregated material. This precaution was necessary to reduce the amount of material above 88000 molecular weight on dodecyl sulfate acrylamide gels which represents intermolecular cross-linking of aggregated protein rather than intramolecular cross-linking. The troponin was diluted in the above buffer to the desired concentrations (0.3–1 mg/ml) and EGTA or $CaCl_2$ was added to 0.1 or 0.01 m*M*, respectively. The dimethylimido esters were dissolved immediately before use in 1.0 *M* triethanolamine at pH 9.0 to give a concentration of 20 mg/ml. The reaction was started by addition of sufficient reagent to give a final concentration of 1 mg/ml in the reaction mixture. The molar excess of imido ester groups with dimethyl suberimidate was 7- to 22-fold assuming 103 mol of lysine per 10^5 g of troponin. With the other bifunctional reagents the excess was greater since they have lower molecular weights. The final pH was 8.5 and did not change throughout the incubation. The protein was maintained at 25°C in a temperature-regulated water bath. The reaction was terminated by dropwise addition of acetic acid (1 *M*) to lower the pH to 7 or below in order to inactivate the reagent. The troponin was dialyzed against pyridine–acetate buffer (pH 6.5) (pyridine–acetic acid–water, 25:1:225 by volume diluted 1:20) and 0.5 m*M* dithiothreitol, and concentrated by freeze drying. The protein was dissolved in the desired buffer, dialyzed, and clarified by centrifugation before use. The cross-linked troponin appeared to be less soluble at low ionic strength than unmodified troponin.

When troponin cross-linked with dimethyl suberimidate was compared with troponin amidinated with methyl acetimidate, the molarity of the reactive imido ester groups was the same (dimethyl suberimidate, 1 mg/ml; methyl acetimidate, 0.8 mg/ml). For comparison, the data presented in Tables I and II and Figure 3 are all of modified troponin where the reaction was terminated after 1 hour.

ATPase Measurements. ATPase activity was measured at 25°C, pH 7.6, in a pH-Stat with an automatic titrator (Titrigraph Type SBR2C, Titrator Type 11, Autoburette ABU 11, Radiometer, Copenhagen). NaOH (0.02 *M*) was

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid: TNBS, 2,4,6-trinitrobenzenesulfonic acid: A, actin; TM, tropomyosin; TN-C, troponin C; TN-I, troponin-I; TN-T, troponin-T.

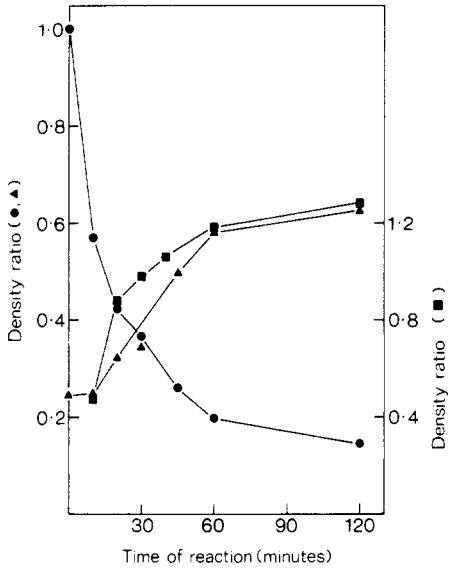

FIGURE 1: Time course of cross-linking of troponin with dimethyl suberimidate. The conditions of cross-linking are described in Materials and Methods. Troponin concentration, 0.3 mg/ml. At designated times 2 ml of reaction mixture was removed and added to 0.5 ml of 1 *M* acetic acid to inactivate unreacted reagent, and dialyzed against pyridine–acetate buffer (pH 6.5) and 0.25 m*M* dithiothreitol and lyophilized. After boiling in 100 μl of electrophoresis sample buffer, 50 μg was electrophoresed on 10% dodecyl sulfate acrylamide gels which were stained in Coomassie Blue. The gels were densitometered but under these loading and staining conditions the measurements can be used for qualitative comparison but not for stoichiometry. Calcium had no measurable effect on cross-linking and the measurements for Ca^{2+} and EGTA samples were averaged. (●) Density ratio of uncross-linked troponin to total troponin where total troponin = troponin-T + -I + -C + cross-linked bands; (■) density ratio of 88000 band to 58500 band; (▲) density ratio of troponin-C to troponin-T + -I + -C (uncross-linked troponin).

used as a titrant. It is generally assumed that 1 μmol of NaOH added represents 1 μmol of ATP hydrolyzed to ADP + P_i (Green and Mommaerts, 1953). Experimental details are described in Table II.

Molecular Weight Determination. Unmodified troponin and troponin which had been cross-linked for 1 hr with dimethyl suberimidate as described above were dialyzed against 1 *M* NaCl, 0.01 *M* imidazole (pH 7.0), and 0.5 m*M* dithiothreitol and sedimented for 6 hr at 80000*g* to remove high molecular weight aggregated material. The dodecyl sulfate acrylamide gels and schlieren pattern from the sedimentation velocity run in the Spinco Model E ultracentrifuge showed there was very little aggregated material. The molecular weights of these troponin preparations were determined by low speed sedimentation equilibrium in an MSE Analytical ultracentrifuge Mk II fitted with an ultraviolet scanner. Sedimentation was continued at 8500 rpm, 5°C, until successive scans at 280 nm taken 24 hr apart were identical. The base line for each cell was determined by subsequent sedimentation at 55000 rpm and then a final scan at low speed again. The weight average molecular weight for each concentration was calculated from a computer fit of the least-squares line to a plot of ln *c* against r^2 for successive sets of 11 data points (taken from about 40 points per scan trace) and the average concentration for each molecular weight was also calculated. Regression lines were fitted to the graphs of $1/M_{app}$ against *c* and best values for $\bar{M}_w$ calculated by extrapolation to zero concentrations. A partial specific volume of 0.73 was assumed for both the control and the cross-linked samples.

Binding of Proteins to Actin–Tropomyosin. Cosedimentation of troponin with actin–tropomyosin was carried out as previously described (Hitchcock et al., 1973). Experimental details are described in Figure 3.

Affinity Chromatography. Rabbit tropomyosin (gift of Dr. Murray Stewart) was covalently linked to cyanogen bromide activated Sepharose 4B (Pharmacia. 10 mg of protein/g of CNBr Sepharose 4B) as previously described (Hitchcock, 1975). The tropomyosin-Sepharose was packed in a 1 × 6 cm column and run at 4°C. Troponin (4–8 mg in 1–1.5 ml) was applied to the column (containing about 20 mg of tropomyosin) equilibrated in 0.15 *M* NaCl, 0.01 *M* imidazole (pH 7.0), 2 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol, and washed in the same buffer, Troponin was eluted with a gradient of 0.15–1.0 *M* NaCl (40 ml each) in the same buffer. Then 6 *M* urea, 0.01 *M* imidazole (pH 7.0), and 0.5 m*M* dithiothreitol was applied to remove any remaining protein (negligible). The protein in the peaks was analyzed by dodecyl sulfate acrylamide gel electrophoresis.

Calcium binding was measured by equilibrium dialysis with the free calcium ion concentration controlled by CaEGTA buffers as previously described (Hitchcock et al., 1973) in the following buffer: 0.2 *M* NaCl, 0.01 *M* imidazole, 2 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol. Samples were counted in a Nuclear Chicago Unilux II liquid scintillation counter.

Acrylamide Gel Electrophoresis. Dodecyl sulfate acrylamide gel electrophoresis was carried out according to Shapiro et al. (1967) and Weber and Osborn (1969) with the modification that the running buffer was 0.1 *M* Tris-bicene (pH 8.3) and 0.1% sodium dodecyl sulfate. Samples were combined with an equal volume of 1.0% sodium dodecyl sulfate, 0.01 *M* Tris-bicene, 10% β-mercaptoethanol, 20% glycerol, and Bromophenol Blue, and immediately boiled for 1–2 min before application to gels. Gels were run at 70 V until the tracking dye reached the bottom of the gels. Gels were stained in 0.5% Coomassie Blue as previously described (Hitchcock et al., 1973) and densitometered on a Joyce Loebl Chromoscan.

Colorimetric Assays. Protein concentration was determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard. Free lysine content of troponin was estimated by reaction with TNBS (Sigma) according to Habeeb (1966).

Chemicals. All chemicals were reagent grade and deionized water was used in making all solutions. Ammonium sulfate and urea were special enzyme grade from Schwarz/Mann. $^{45}CaCl_2$ was obtained from Amersham.

Results

Cross-Linking of Troponin with Dimethyl Suberimidate. Conditions for cross-linking troponin were first investigated using dimethyl suberimidate. Rabbit troponin was cross-linked for increasing times to determine the minimal time for complete cross-linking (Figures 1 and 2). The dodecyl sulfate acrylamide gels of cross-linked protein show that the three troponin bands decrease in intensity and two major cross-linked bands (58500 and 88000 in molecular weight) appear with time (●). Cross-linking is virtually complete after 1 hr at 25°C. The 58500 band appears before the 88000 band (■). Troponin-C does not become fully cross-linked to the other troponin components even after 4 hr (▲). The time course of cross-linking is the same in the presence and absence of calcium (with 2 m*M* $MgCl_2$). The measurements in Figure 1 cannot be used for estimation of stoichi-

ometry since they were from gels stained with Coomassie Blue and the cross-linked bands are in the nonlinear range.

Although cross-linking of troponin is nearly complete after 1 hr, not all the lysines are modified. When free lysines are reacted with TNBS according to Habeeb (1966), cross-linked troponin contains more free lysine than amidinated troponin. (Absorbance at 335 nm/mg of protein for unmodified troponin = 9.5–11, cross-linked troponin = 7.5–8.4, bovine serum albumin (unmodified, for a standard) = 6.5.) Amidination of troponin with methyl acetimidate is incomplete at 1 hr and takes 2–3 hr to reach completion in these conditions. (Absorbance at 335 nm/mg of protein, 1 hr = 6.3, 3 hr = 0.7–2.0.) The TNBS reaction has not allowed calculation of the number of moles of free lysine and in my hands gives somewhat variable values. It is useful only as a rough measure of free lysine.

When cross-linked troponin is electrophoresed on 7.5% dodecyl sulfate acrylamide gels calibrated with standard proteins, the molecular weights of the major cross-linked bands are estimated to be 88000 and 58500. The molecular weight of the troponin complex is about 80000, close to that of the slower migrating band. Analysis of troponin with cleavable cross-linkers has shown that the 88000 band is troponin-T + -I + -C and the 58500 band is primarily troponin-T + -I (Hitchcock and Lutter, 1975).

The weight average molecular weights of troponin and cross-linked troponin, determined by the method of low speed sedimentation equilibrium, are the same: troponin, 80000 ± 1900; cross-linked troponin, 80600 ± 2600. This assures that the cross-linking observed is intramolecular rather than intermolecular. It also shows that the 88000 band on gels is not due to cross-linking between molecules (for example, the troponin-I + -C of one molecule and troponin-T of a neighbor). The assumed $\bar{V}$ of 0.73 is reasonable since the control troponin gave a molecular weight close to that expected (Hartshorne and Driezen, 1973).

Cross-Linking of Troponin with Different Length Dimethylimido Esters. Rabbit troponin was reacted with the following dimethylimido esters where the hydrocarbon chains between the terminal carbons range from $(CH_2)_2$ to $(CH_2)_6$: dimethyl succinimidate ($(CH_2)_2$, 0.62 nm), dimethyl adipimidate ($(CH_2)_4$, 0.87 nm), dimethyl pimelimidate ($(CH_2)_5$, 1.00 nm), and dimethyl suberimidate ($(CH_2)_6$, 1.12 nm). Using bond lengths and angles from Pauling (1960), the lengths calculated are between reactive nitrogens on cross-linked lysines in the fully extended configuration of these cross-linkers. Dodecyl sulfate acrylamide gels of troponin cross-linked with these reagents are shown in Figure 2. With the shortest cross-linker, no major band corresponding to a complex of troponin-T + -I + -C (88000) is found. However, limited cross-linking between troponin-T + -I (58500) is observed. With dimethyl adipimidate some of the protein is found in the 88000 band. With dimethyl pimelimidate and dimethyl suberimidate, cross-linking into the two major bands is virtually complete, but there is always some troponin-C which remains. If care is taken to remove aggregated material by centrifugation before cross-linking and the protein is dilute, there is very little density in the gels above the 88000 molecular weight band. No difference between EGTA and Ca^{2+} in the presence of 2 m*M* $MgCl_2$ has been observed with any cross-linker, even at short cross-linking times (20 min). (Densitometric measurements of gels would allow detection of differences of about 10–20% or greater.)

As a control, troponin was reacted with the monovalent

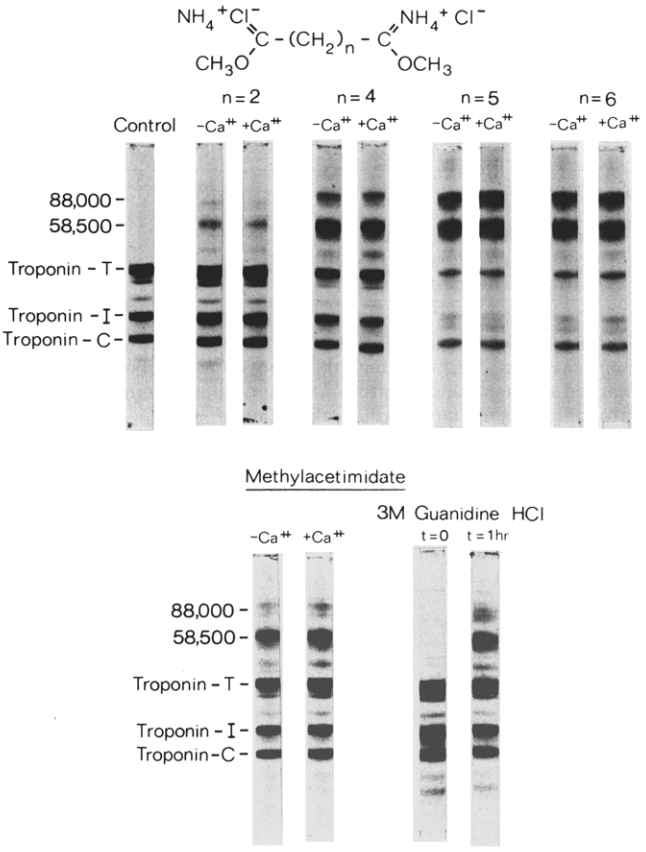

FIGURE 2: Cross-linking of troponin with different length dimethylimido esters. Dodecyl sulfate acrylamide gels (10%) of cross-linked troponin are stained in Coomassie Blue. Troponin (0.68 mg/ml) was cross-linked for 1.5 hr at 25°C in the conditions described in Materials and Methods with the following reagents: methyl acetimidate (0.8 mg/ml), dimethyl succinimidate, dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate (all 1 mg/ml). With dimethyl succinimidate and dimethyl adipimidate, cross-linking was nearly complete at 20 min. With dimethyl pimelimidate and dimethyl suberimidate cross-linking was complete by 1 hr; 30–55 μg of protein was applied to the gels. In the experiment with methyl acetimidate solid guanidine hydrochloride (Mann) was added to 5 *M* before addition of reagent or after 1 hr. At this time additional reagent (plus 0.8 mg/ml) was added to these tubes and the reaction was allowed to proceed for 3 more hr.

reagent, methyl acetimidate. In amidinated troponin additional bands are observed which comigrate with those of cross-linked troponin (Figure 2). These bands represent cross-linking since they are not removed when the protein subunits are dissociated in 6 *M* urea or 3 *M* guanidine hydrochloride. When amidination is carried out in the presence of 3 *M* guanidine hydrochloride, the cross-linked bands are not present. The reason for cross-linking of troponin with a monovalent reagent is not understood. Cross-linking with methyl acetimidate has been observed with three preparations of reagent synthesized in different laboratories (S. E. Hitchcock and J. Coggins).

On the basis of the experiments with different length dimethylimido esters, it has been concluded that all three troponin components have lysines which are not greater than 0.9–1.0 nm from each other and that troponin-T and -I have lysines which are closer to each other, 0.6–0.7 nm or even less if the methyl acetimidate is in some way cross-linking. The proximity of the troponin subunits may be underestimated using dimethyl succinimidate since treatment of troponin with another protein cross-linker of similar length (0.6 nm, tartryl diazide) shows virtually complete

Table I: Calcium Binding by Modified Troponin.[a]

	Calcium Bound (μmol g^{-1})	
	$[Ca^{2+}] = 3 \times 10^{-7} M$	$[Ca^{2+}] = 3 \times 10^{-6} M$
Control troponin	23.28	30.44
	25.44[b]	31.79[b]
Amidinated troponin, EGTA	24.87	30.82
Cross-linked troponin, EGTA	21.87	33.04
Cross-linked troponin, Ca^{2+}	26.96	36.05

[a] Calcium binding was measured by equilibrium dialysis against ^{45}CaEGTA buffers as described in Materials and Methods. Troponin concentrations were 3–8 mg/ml. [b] Control troponin which had been carried through the same preparation procedures as the modified troponins has retained normal calcium binding.

Table II: Effect of Modification of Troponin on Calcium Sensitivity.[a]

	Troponin/ Tropomyosin Molar Ratio	Specific Activity (μmol of H^+ per mg of myosin per min) EGTA	$+Ca^{2+}$	Calcium Sensitivity (%)
No troponin	0	0.458	0.472	3.0
Control troponin	0.65	0.063	0.414	84.8
	0.65	0.097[b]	0.465[b]	79.1[b]
Amidinated troponin	0.65	0.188	0.410	54.1
	2.58	0.160	0.326	50.9
Cross-linked troponin, EGTA	0.65	0.382	0.396	3.5
	3.68	0.319	0.368	13.3
Cross-linked troponin, Ca^{2+}	0.65	0.354	0.382	7.3
	3.68	0.218	0.333	34.5

[a] Assay conditions: 36 mM NaCl, 2 mM $MgCl_2$, 0.7 mM ATP, 0.1 mM EGTA, $+Ca^{2+}$, with 0.2 mM $CaCl_2$, pH 7.6, 25°C. Each assay contained 0.72 mg of rabbit myosin, 0.18 mg of rabbit F-actin, 0.06 mg of chicken tropomyosin, troponin as indicated assuming 80000 molecular weight, in 10 ml. The proteins were combined in the following order: actin, tropomyosin, troponin, brought to 0.6 M NaCl, myosin. This mixture was added dropwise into the ATPase solution and the reaction started by addition of 0.7 ml of 10 mM ATP. Calcium sensitivity = [1 – (EGTA ATPase/Ca^{2+} ATPase)] 100. [b] Control troponin which had been carried through the same preparation procedures as the modified troponins has retained normal calcium sensitivity.

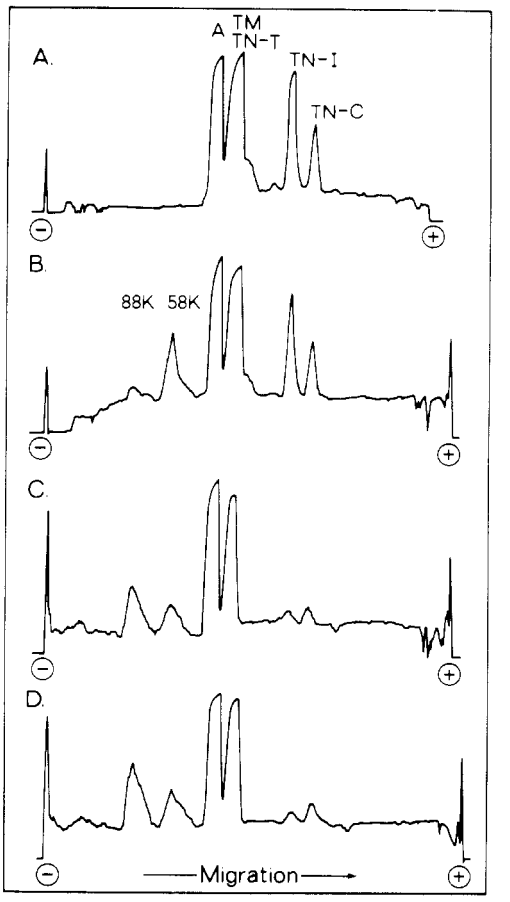

FIGURE 3: Binding of modified troponin to actin-tropomyosin. Troponin was sedimented in 0.2 M NaCl, 10 mM imidazole, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 mM EGTA or 0.1 mM CaEGTA for 3 hr at 80000g to remove any aggregated material. The supernatant was combined with F-actin–tropomyosin and sedimented as before. The pellet was rinsed, resuspended in the same buffer, and sedimented a second time. Final protein concentrations were actin, 0.5 mg/ml; tropomyosin, 0.17 mg/ml; troponin, 0.25, mg/ml; final volume, 2 ml. The densitometer tracings are of 10% dodecyl sulfate acrylamide gels of the actin–tropomyosin–troponin pellets stained in Coomassie Blue. The – marks the top, the + the bottom of the gel. The binding was the same with and without calcium and the gels shown are of binding in 0.1 mM EGTA. (A) Control troponin; (B) amidinated troponin; (C) troponin cross-linked with dimethyl suberimidate in 0.1 mM EGTA; (D) troponin cross-linked with dimethyl suberimidate in the presence of 0.01 mM $CaCl_2$.

cross-linking into the 58500 and 88000 bands (Hitchcock and Lutter, 1975). Although small amounts of the troponin-I + -C (42000) or troponin-T + -C (55000) complexes are present, these are not major products of cross-linking.

Effect of Cross-Linking on the Function of Troponin. The function of troponin which had been cross-linked with dimethyl suberimidate was investigated. As a control troponin was amidinated with methyl acetimidate in order to distinguish the effect of amidination alone from that of cross-linking. The troponin was cross-linked for 1 hr to obtain nearly complete cross-linking with minimal further modification of lysine.

The calcium binding by cross-linked and amidinated troponin was found to be the same as unmodified troponin (Table I). Troponin which has been amidinated for 3 or 4 hr in the presence and absence of calcium also binds calcium normally.

Although cross-linked troponin binds calcium normally, it does not confer calcium sensitivity on the actomyosin ATPase (Table II). At stoichiometric amounts, it is nearly completely lost. With greater amounts of cross-linked troponin, low levels of calcium sensitivity are observed. The calcium sensitivity of amidinated troponin is reduced relative to the control. Some of the loss of calcium sensitivity must be due to amidination rather than cross-linking since the small amount of cross-linking observed cannot account for the large loss of sensitivity.

The binding of cross-linked troponin to actin–tropomyosin by cosedimentation was measured (Figure 3). Densitometric measurements of the gels show that the troponin–tropomyosin to actin ratio was the same with unmodified, amidinated, and cross-linked troponin. Calcium had no effect on the binding.

The binding of modified troponin to tropomyosin was studied by affinity chromatography. Rabbit tropomyosin was covalently linked to CNBr-activated Sepharose 4B (Pharmacia). Unmodified, amidinated, and cross-linked troponin all bound to the tropomyosin-Sepharose and were eluted at approximately the same NaCl concentration (0.3 M) (experimental details in Materials and Methods).

These experiments show that although cross-linking de-

stroys the calcium sensitivity of troponin more than amidination alone, the binding of modified troponin to tropomyosin and actin–tropomyosin is indistinguishable from unmodified troponin.

Discussion

Cross-linking troponin with different length dimethylimido esters allows a choice between two schematic models of troponin structure: TCI, where the subunits are in an elongated arrangement, and $\overset{\text{TI}}{\text{C}}$, where they are compact. We know from a variety of studies that troponin-C interacts with troponin-I and troponin-T (see introduction). Because troponin-I and -T are so insoluble, it has been difficult to study their interaction. A major new finding from this study is that troponin-I and -T are close to each other, 0.6 nm or less, in the native troponin complex. This conclusion is based on the observation that the shortest cross-linking reagent gives a troponin-T + -I band and the evidence that they are formed first when cross-linked with dimethyl suberimidate. It does not prove that they interact with each other. Since the major products of cross-linking are complexes composed of troponin-T + -I and T + I + C, model $\overset{\text{TI}}{\text{C}}$, where all three components are close to each other, is more acceptable than model TCI. The details of course will not be known until the three-dimensional structure of troponin is solved.

Calcium has no detectable influence on cross-linking or amidination using the probes employed in this study. The different length cross-linkers give the same result in the presence and absence of calcium. Although the maximal lengths of the reagents are listed in the text, it should be realized that the minimal lengths are zero since there is free rotation around the carbon–carbon bonds.

Cross-linking with dimethyl suberimidate destroys the ability of troponin to confer calcium sensitivity on the actomyosin ATPase. However, calcium binding, cosedimentation with actin–tropomyosin, and binding to a tropomyosin affinity column are all apparently normal. Amidination with imido esters preserves the net charge of the protein. Possibly for this reason the modified troponin can interact with actin and tropomyosin. The change in position of the charge on the protein and any conformational change in cross-linked protein may prevent troponin from interacting with actin, tropomyosin, and calcium in the very specific way required for calcium-sensitive inhibition.

The cross-linking observed when troponin was modified with methyl acetimidate is surprising and not understood. This was intended as a control to distinguish the effect of cross-linking from amidination alone.

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