Effect of Calcium(II) on the Interaction between the Subunits of Troponin and Tropomyosin†

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ABSTRACT: The method of M. Greaser and J. Gergely for the isolation of the subunits of troponin was improved. The subunits are pure as judged by sodium dodecyl sulfate gel electrophoresis. The interactions between the subunits of troponin, between these subunits and tropomyosin, and the effect of Ca^{2+} on these interactions were studied by normal disc gel electrophoresis, ultracentrifugation, and fluorescence measurements. The Ca^{2+} binding subunit of troponin (TN-C) binds to both other subunits of troponin, *i.e.*, the tropomyosin-binding subunit (TN-T) and the subunit that inhibits the ATPase activity of actomyosin (TN-I). TN-T as well as TN-I are modified by Ca^{2+} *via* TN-C. TN-T changes tropomyosin to a state where the complex of TN-C–TN-I can exert its action on tropomyosin. A model is presented to show how the troponin–tropomyosin system is organized and influenced by Ca^{2+}.

Troponin is a protein located on the thin filaments of muscle. It constitutes together with tropomyosin, another protein of the thin filaments, the system that regulates the interaction between actin and myosin (Ebashi and Endo, 1968). Upon stimulation of the nerve, Ca^{2+} is released from the sarcoplasmic reticulum, a vesicular system surrounding the myofibrils, and binds to troponin. This binding induces a structural change in troponin which is thought to be propagated *via* tropomyosin to actin (Ebashi *et al.*, 1968; Tonomura *et al.*, 1969). As a result, actin interacts with myosin and the muscle contracts. This model for the regulation of muscular contraction has been complicated by the recent finding that troponin is not a single molecule but is composed of three subunits (Greaser and Gergely, 1971; Drabikowski *et al.*, 1971a; Ebashi *et al.*, 1971). The first subunit TN-C[1] is the true Ca^{2+} binding component. It undergoes a structural change when it binds Ca^{2+} (van Eerd and Kawasaki, 1972; Murray and Kay, 1972). The second subunit TN-I inhibits the interaction between myosin and actin and as such can be called the repressor of muscular contraction. The third subunit TN-T has a strong affinity for tropomyosin. Several laboratories have succeeded in isolating the subunits of troponin, though it seems that the methods used are not always satisfactory. Moreover, it is not clear how these subunits interact and together regulate muscular contraction. In this paper we describe an improved method for the isolation of the subunits of troponin which are pure as judged by sodium dodecyl sulfate gel electrophoresis. We also report on the interactions between these subunits and we present a model of the organization of the troponin–tropomyosin system, in the state of activation as well as relaxation.

Experimental Section

Muscle Proteins. Troponin and tropomyosin were prepared from rabbit skeletal muscle according to the method of Ebashi *et al.* (1968). The purity of these proteins was checked by sodium dodecyl sulfate gel electrophoresis. If necessary, troponin was further purified by applying it on a column packed with DEAE-cellulose equilibrated with 20 mM Tris-HCl (pH 7.5) and eluting it with a linear KCl gradient (from 0 to 0.4 M) in the same buffer solution. Troponin was eluted at 0.22 M KCl (see Results and Discussion). DEAE-cellulose chromatography was performed in a cold room.

Troponin was separated into its subunits by applying it on a column packed with DEAE-Sephadex A-25 equilibrated with 6 M urea–20 mM Tris-HCl (pH 8.5)–2 mM β-mercaptoethanol at room temperature (see Results and Discussion). The subunits were eluted with a linear gradient of 0–0.6 M KCl in the same buffer. TN-I was not retained by the DEAE-Sephadex. TN-T eluted at 0.17 M KCl and TN-C at 0.4 M KCl. After separation, TN-C and TN-I were dialyzed against 0.3 mM bicarbonate, TN-T against 0.3 mM bicarbonate–0.3 M KCl. Upon dialysis, TN-I often became slightly turbid in which case it was clarified by centrifugation. Urea was purified by passing it as an 8 M solution over a mixed bed ion-exchange resin, *e.g.*, Amberlite MB-3 (Hirs, 1967).

Gel Electrophoresis. Standard disc gel electrophoresis was performed according to the method of Davis (1964) using 7% gels in 100 × 5 mm tubes. The buffer system used was Tris-glycine (pH 8.3). Protein (25–50 μg) was applied to each gel. For gel electrophoresis in the absence of Ca^{2+}, 0.5 mM EGTA[1] was added to the gels and the chamber buffer. Electrophoresis was conducted at 3.5 mA/tube until the marker dye (Bromophenol Blue) migrated to within 1 cm from the end of the tubes. The gels were stained for 2 hr in a solution of Coomassie brilliant Blue (0.12% Coomassie Blue–45% methyl alcohol–9% acetic acid). Destaining was performed by repeated washings with a solution containing 5% methyl alcohol and 7.5% acetic acid.

Sodium dodecyl sulfate gel electrophoresis was performed according to Weber and Osborn (1969) using 10% gels and a buffer of 20 mM sodium phosphate (pH 7.0). Samples were incubated with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 2 min in boiling water. Electrophoresis was conducted at 5 mA/tube (tubes 100 × 5 mm) for 2–2.5 hr. Staining

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[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DNS, 1-dimethylaminonaphthalene-5-sulfonyl chloride; OD, optical density; TN-C, Ca^{2+} binding subunit of troponin; TN-T, tropomyosin-binding subunit; TN-I, subunit that inhibits ATPase activity of actomyosin.

and destaining were the same as in the case of normal gel electrophoresis.

Fluorescence Measurements. A Hitachi MPF-2A fluorescence spectrophotometer was used for fluorescence measurements. DNA fluorescence was observed at 496–520 nm after excitation at 340 nm. Polacoat ultraviolet polarizing filters were used for measuring the degree of fluorescence polarization. The degree of fluorescence polarization, P, is defined as

$$P = \frac{(I_{//} - I_{\perp})}{(I_{//} + I_{\perp})}$$

where $I_{//}$ is the fluorescence intensity with the analyzer parallel to the polarizer and $I_{\perp}$ is the fluorescence intensity with the analyzer perpendicular to the polarizer. All measurements were performed at 30°. The OD_{250} values of the samples were observed frequently to make sure that observed changes in fluorescence were not caused by aggregation. The OD values of the samples at the excitation wavelength were less than 0.08 for DNS fluorescence measurements.

Fluorescence Labeling. Tropomyosin and the subunits of troponin were labeled with the fluorescent probe DNS in 10 mM $NaHCO_3$–0.1 M KCl (0.3 M KCl in case of TN-T; 2 mM β-mercaptoethanol was added in case of TN-I) at 0°. The mixture was left standing for 12 hr and dialyzed against 0.5 mM $NaHCO_3$–0.1 M KCl (0.3 M KCl in case of TN-T; 2 mM β-mercaptoethanol was added in case of TN-I). The molar ratio of DNS to protein in the solution after dialysis was less than 1:2 (before dialysis this ratio was 2.5:1).

Free Ca^{2+} concentrations were adjusted using a Ca buffer of Ca^{2+} and EGTA in 30 mM sodium cacodylate buffer (pH 6.9). Free Ca^{2+} concentrations were calculated according to Schwartzenbach *et al.* (1957). Corrections were made for pH changes due to the addition of Ca^{2+}.

Ultracentrifugation. $s_{20,w}$ values were determined in a Spinco Model E ultracentrifuge equipped with schlieren optics; solvent conditions: 0.3 M KCl–0.3 mM $NaHCO_3$; temperature, 25°; 0.5 mM EGTA was added to obtain the minus-Ca^{2+} state. Protein concentrations ranged from 5 to 10 mg/ml. s values were not extrapolated to zero protein concentration; rotation speed, 53,000 or 59,000 rpm.

Protein concentrations were determined by the biuret method of Gornall *et al.* (1949).

Results

Purification of Troponin and Isolation of Subunits. Because some subunits of troponin, especially TN-T, are very susceptible to proteolytic degradation (Drabikowski *et al.*, 1971b), most preparations of troponin usually contain less than equimolar amounts of TN-T and a number of degradation products. One of these degradation products with a molecular weight of 14,000 has been described in some detail (Drabikowski *et al.*, 1971b; Wilkinson *et al.*, 1971). These degradation products have to be removed before it is possible to isolate the subunits of troponin in a pure form.

Purification. For the removal of degradation products of troponin we used DEAE-cellulose chromatography. In Figure 1 is shown the elution pattern after DEAE-cellulose chromatography. The solid line and the dashed line represent the elution patterns of two different troponin preparations. There is a small amount of material that does not get attached to the DEAE-cellulose. It is probably the γ component of Lee and Watanabe (1970). Troponin elutes in two peaks. When the peaks are analyzed by sodium dodecyl sulfate gel

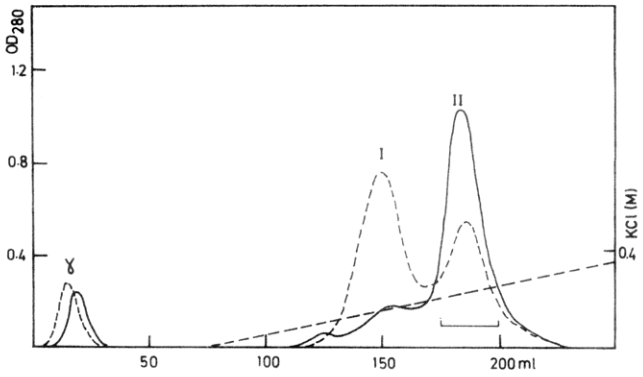

FIGURE 1: Purification of troponin by DEAE-cellulose chromatography: 91 mg (——) or 103 mg (----) of crude troponin was loaded on a column (40 × 1.3 cm) of DEAE-cellulose equilibrated with 20 mM Tris-HCl (pH 7.5). The column was eluted with 75 ml of starting buffer followed by a gradient of 0–0.4 M KCl (200 ml) in the same buffer. Peak II corresponds to pure troponin. The solid line and the broken line correspond to two different troponin preparations. A solid bar indicates the fraction collected and used for the isolation of subunits.

electrophoresis (Figure 2), it can be seen that peak I lacks TN-T. Besides TN-I and TN-C, this peak also contains large amounts of other proteins. If we assume a molecular weight of 37,000 for TN-T, 23,000 for TN-I, and 18,000 for TN-C (Perry, 1971), we can estimate the molecular weights of the unknown proteinous material. Their estimated molecular weights are 29,000 and 14,000. Peak II contains only the three subunits of troponin and it contains no proteins with a molecular weight of 29,000 and 14,000. The relative height of peaks I and II depends on the preparation. This may indicate that peak I contains partially degraded troponin and that the 29,000 and 14,000 dalton components are degradation products of troponin. Fairly often the TN-I component, even in

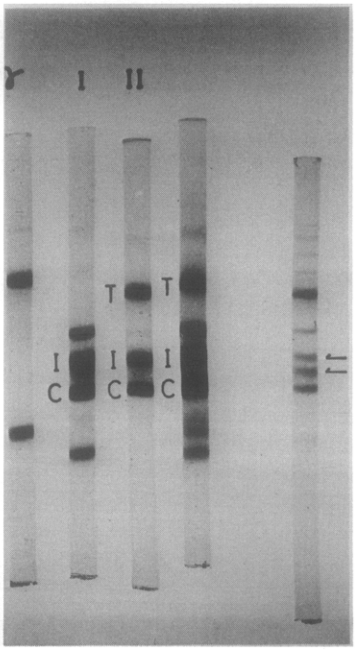

FIGURE 2: Analysis of the peaks eluted during the purification of troponin on DEAE-cellulose (Figure 1). γ, I, and II correspond to the peaks in Figure 1. The fourth gel corresponds to crude troponin before DEAE-cellulose purification. The utmost right gel shows that at low protein concentration the TN-I band in crude troponin preparations sometimes splits into two bands.

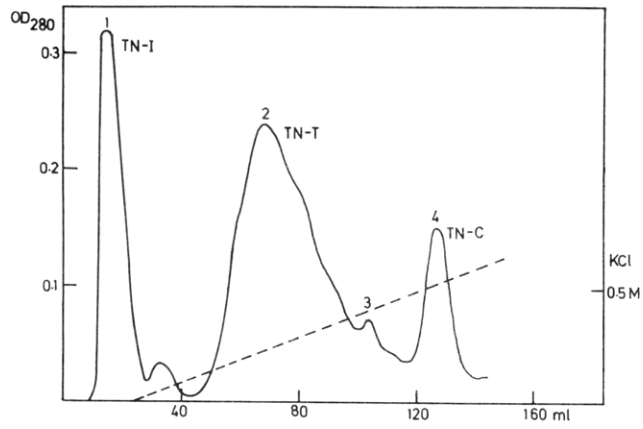

FIGURE 3: Isolation of the subunits of troponin: 40 mg of purified troponin was loaded on a column (22 × 1.1 cm) of DEAE-Sephadex A-25 equilibrated with 6 M urea–20 mM Tris-HCl (pH 8.5) containing 2 mM β-mercaptoethanol. The column was eluted with 25 ml of starting buffer followed by a gradient of 0–0.6 M KCl (120 ml) in the same buffer. The peaks were identified by sodium dodecyl sulfate gel electrophoresis (Figure 4).

DEAE-cellulose purified troponin, shows a broad band in sodium dodecyl sulfate gel electrophoresis and in long gels and at a low protein concentration this band splits into two bands (see Figure 2, last gel). Only peak II is used for the isolation of subunits.

Isolation of Subunits. To separate troponin into its subunits, we slightly modified the method of Greaser and Gergely (1971). We used DEAE-Sephadex A-25 instead of DEAE-Sephadex A-50. In Figure 3 is shown the elution pattern of DEAE-cellulose purified troponin on DEAE-Sephadex A-25 in the presence of 6 M urea. Sodium dodecyl sulfate gel electrophoresis of the peaks is shown in Figure 4. DEAE-Sephadex A-25 gives a slightly different elution pattern from

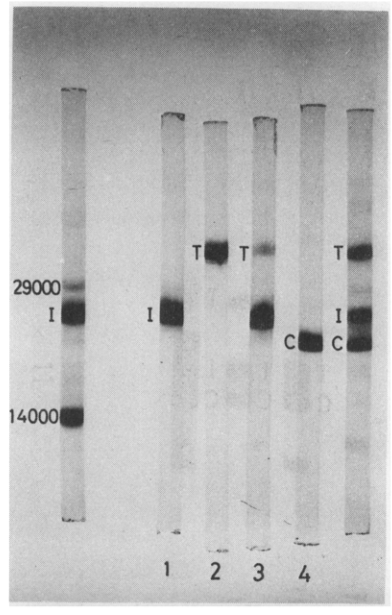

FIGURE 4: Analysis of the peaks from Figure 3 by sodium dodecyl sulfate gel electrophoresis. The numbers correspond with the peaks in Figure 3. The utmost right gel is the original sample of troponin before separation into subunits. The utmost left gel is an example of peak I after separation of unpurified troponin. It shows TN-I contaminated with proteins of 29,000 and 14,000 molecular weight. The small peak eluted between TN-T and TN-C contains protein with a molecular weight very similar to that of TN-I.

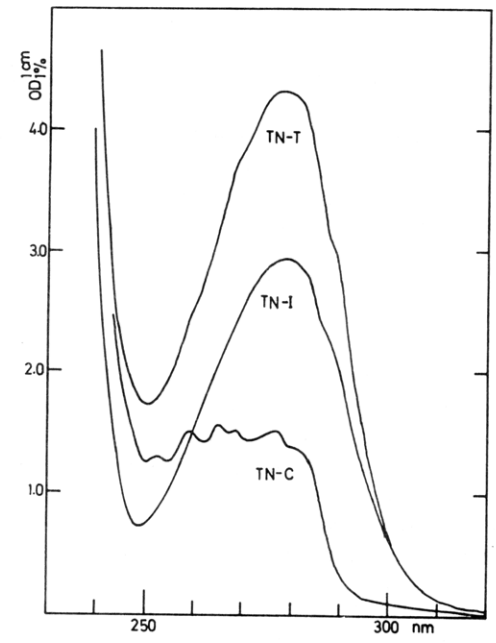

FIGURE 5: Ultraviolet spectra of 1% solutions of the subunits of troponin. The optical path length is 1 cm. Solvent conditions: 0.3 mM bicarbonate in TN-C and TN-I–0.3 mM bicarbonate–0.3 M KCl in TN-T.

A-50, its main advantage being a clear separation of TN-I and TN-T. In Greaser's method the components with molecular weights 14,000 and 29,000 are not retained by the Sephadex and TN-I is eluted at low ionic strength closely followed by TN-T. In the case of DEAE-Sephadex A-25, TN-I is not retained by the Sephadex and elutes together with the 14,000 and 29,000 molecular weight components (see Figure 4, first gel). Therefore it is essential to remove these degradation products first by DEAE-cellulose chromatography. At 0.3 M KCl, a peak elutes which contains a protein with a molecular weight very close to that of TN-I. TN-T is eluted at 0.17 M KCl and TN-C at 0.45 M KCl.

To summarize, when troponin is first purified on DEAE-cellulose, its subunits can be readily separated by DEAE-Sephadex A-25 column chromatography in the presence of 6 M urea.

Optical Properties of the Subunits of Troponin. In Figure 5 are presented the uv absorption spectra of 1% solutions of the individual components of troponin. The spectrum of TN-C shows that this component does not contain tryptophan, which has been reported before by Greaser and Gergely (1970). The peak at 276 nm and the shoulder at 282 nm are due to tyrosine residues. The peaks at 268, 265, 259, and 253 nm are due to phenylalanine residues in TN-C. The number of tyrosine and tryptophan residues per subunit is estimated from the spectra of the subunits assuming a molecular weight of 37,000 for TN-T, 23,000 for TN-I, and 18,000 for TN-C and assuming a molar extinction coefficient at 280 nm of 1280 for the tyrosine residues and 5600 for the tryptophan residues (Handbook of Biochemistry, Cleveland, Ohio, The Chemical Rubber Co., 1968, p B-18). It should further be taken into account that the total number of tryptophan residues as estimated by Arai and Watanabe (1968) and Staprans and Watanabe (1970) is 4 per 100,000 daltons. Amino acid analysis performed by Ebashi *et al.* (1972) shows four tyrosine residues in TN-T, two tyrosine residues in TN-I, and two tyrosine residues in TN-C, although no estimation on tryptophan. In Table I are presented the extinction values at 280 nm of 1% solutions of

TABLE I: Uv Characteristics of the Subunits of Troponin.

	$E^{1\%}_{1\text{ cm, }280}$	E^{*} [a]	OD_{280}/OD_{260}	Tyrosine (res/mol)	Tryptophan (res/mol)
TN-T	4.42	4.30	1.69	4	2
TN-I	2.91	3.00	1.85	1	1
TN-C	1.41	1.42	0.94	2	0

[a] E^{*} is the $E^{1\%}_{1\text{ cm, }280}$ value calculated from the number of tyrosine and tryptophan residues shown in this table.

the troponin subunits as well as the OD_{280}/OD_{260} ratios and the estimated tryptophan and tyrosine residues per subunit. The uv absorption spectra suggest one tryptophan and one tyrosine residue per molecule of TN-I although amino acid analyses by Ebashi *et al.* (1972) indicate two tyrosine residues per molecule of TN-I. The tryptophan content of the subunits of troponin was also reported by Greaser and Gergely (1973), but their number for the tryptophan content of TN-I (two residues/molecule) is not in agreement with our results.

Disc Gel Electrophoresis. We examined the electrophoretic patterns of the subunits of troponin, its combinations (Figure 6), and combinations with tropomyosin (Figure 7), in the presence as well as in the absence of Ca^{2+}.

TN-T does not enter the gel in the presence as well as in the absence of Ca^{2+} presumably because it is not soluble at the low ionic strength necessary for electrophoresis.

TN-I does not enter the gel in the presence of Ca^{2+} but it shows a broad distorted band in the absence of Ca^{2+}.

TN-C shows one band migrating with the marker dye, in the presence as well as in the absence of Ca^{2+}.

TN-T–TN-I shows the same electrophoretic pattern as TN-I. If TN-I should bind to TN-T we should expect a different electrophoretic pattern. Therefore it is not likely that TN-T and TN-I interact strongly.

TN-T–TN-C shows a faint band of excess TN-C and another band presumably the complex of TN-T and TN-C. In the absence of Ca^{2+} this pattern becomes more complex. The TN-C band is much stronger and the top of the gel shows some stain. Also the electrophoretic mobility of the complex has been changed. Presumably some TN-C dissociated from TN-T. Therefore we think that the binding between TN-C and TN-T is Ca^{2+} sensitive and that upon removal of Ca^{2+} the binding between TN-C and TN-T becomes weaker.

TN-I–TN-C shows also a faint band of excess TN-C plus another band presumably the complex of TN-I and TN-C. This band disappears in the absence of Ca^{2+} and the TN-C band becomes stronger. On the other hand, two new bands appear. One band has the same electrophoretic mobility as TN-I in the minus-Ca^{2+} state. The other is presumably the complex of TN-I and TN-C in the absence of Ca^{2+}. These gels suggest that the interaction between TN-C and TN-I is Ca^{2+} sensitive and that in the absence of Ca^{2+} this interaction becomes weaker.

Combinations with Tropomyosin. When we look at the same subunits and combinations of subunits in the presence of tropomyosin (Figure 7) we can make the following comments. (1) Tropomyosin associates at low ionic strength and therefore does not show a sharp band but shows a wide smear, which makes it difficult to draw conclusions. (2) TN-C does not bind to tropomyosin. The apparent difference in electrophoretic mobility of TN-C is not real but is due to a difference of the distance of migration of the marker dye. (3) In the gels of tropomyosin–TN-T–TN-C we do not see a band corresponding to the complex TN-T–TN-C, independent of Ca^{2+} concentration. Only a band of excess TN-C is present whose intensity is independent of the Ca^{2+} concentration. Therefore these gels suggest that the complex of TN-T–TN-C binds to tropomyosin in the plus-Ca^{2+} state as well as in the minus-Ca^{2+} state. (4) In the last pair of gels we see in the plus-Ca^{2+} state the band corresponding to the complex of TN-C–TN-I. This indicates that this complex presumably does not bind to tropomyosin in the presence of Ca^{2+}. (5) We cannot draw conclusions from these gels whether TN-I or TN-T and also the complex of TN-I–TN-C in the minus-Ca^{2+} state bind to tropomyosin.

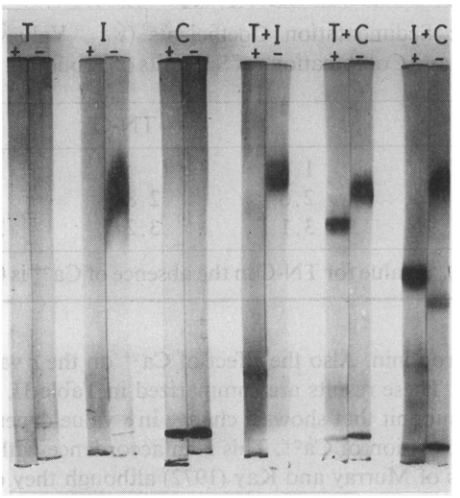

FIGURE 6: Normal disc gel electrophoresis of individual subunits and combinations of subunits of troponin in the presence and absence of Ca^{2+}. T stands for TN-T, I stands for TN-I, and C stands for TN-C. The plus-Ca^{2+} state (+) contains only contaminating trace amounts of Ca^{2+}. The minus-Ca^{2+} state (−) is obtained by chelating free Ca^{2+} by 0.5 mM EGTA incorporated in the gels and the chamber buffer. In combinations the subunits are present in approximately 1:1 molar ratio.

Ultracentrifugation. Apparent sedimentation coefficients were determined for the subunits and the mixtures of the sub-

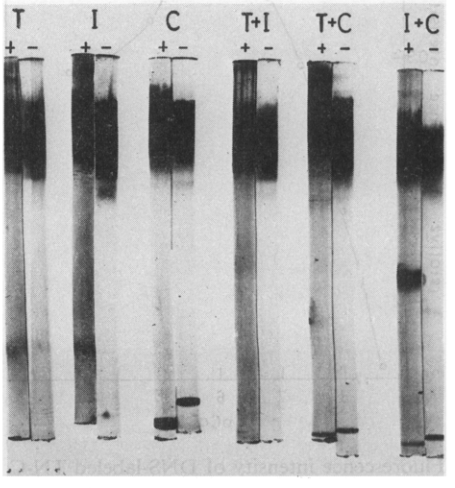

FIGURE 7: Normal disc gel electrophoresis of the same subunits and combinations of subunits of troponin as in Figure 6 but now in the presence of tropomyosin. Other conditions as in Figure 6.

TABLE II: Sedimentation Coefficients ($s_{20,w}$ Values) of the Subunits and Combinations of Subunits of Troponin.

		+ TN-C	+ TN-I
TN-C	1.8[a]		
TN-I	2.6	2.8	
TN-T	3.1	3.2	3.0

[a] The $s_{20,w}$ value for TN-C in the absence of Ca^{2+} is 0.9.

units of troponin. Also the effect of Ca^{2+} on the s values was observed. These results are summarized in Table II. TN-C is the only subunit that shows a change in s value depending on the concentration of Ca^{2+}. This is in accordance with the observations of Murray and Kay (1972) although they observed slightly higher s values. The mixtures of the subunits of troponin all show only one single peak with rather similar s values. These results suggest that TN-C can form a complex with TN-T and also with TN-I, otherwise a second slower moving peak would be expected. The apparent sedimentation coefficients of TN-I, TN-T, and combinations of these subunits are very similar making it virtually impossible to observe two peaks in case these components do not bind. The rather low s value of the mixture suggests, however, that there is no interaction between these two subunits.

Fluorescence Measurements. Fluorescence of Subunits of Troponin and Combinations of Subunits. (1) *DNS Fluorescence.* The subunits of troponin were labeled with the fluorescent probe DNS, and the interactions among the subunits and their Ca^{2+} sensitivity were studied by the DNS fluorescence measurements.

Of the subunits of troponin labeled with DNS, only TN-C^{DNS} showed Ca^{2+} sensitivity. In Figure 8 is presented the DNS fluorescence intensity of DNS labeled TN-C as a function of pCa. There is a large increase in fluorescence intensity when the pCa is lowered from 9 to 6 and a subsequent decrease in intensity when the pCa is further lowered. The increase corresponds to a structural change in TN-C when the high affinity binding sites are loaded with Ca^{2+}. The decrease may correspond to a change in TN-C when Ca^{2+} binding sites with a low affinity for Ca^{2+} are charged with Ca^{2+} (Hartshorne and Pyun, 1971).

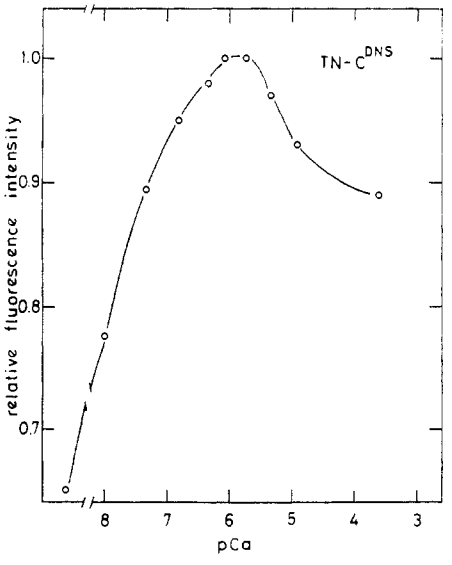

FIGURE 8: Fluorescence intensity of DNS-labeled TN-C as a function of pCa. The protein concentration is 0.1 mg/ml. Solvent condition: 0.1 M KCl–30 mM sodium cacodylate (pH 6.9)–0.9 mM EGTA (varying amount of $CaCl_2$). The fluorescence intensity is expressed as relative fluorescence intensity with SD ±0.01.

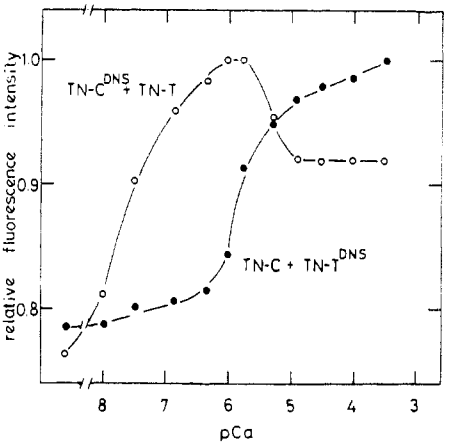

FIGURE 9: Fluorescence intensity of DNS-labeled TN-C (○) and DNS-labeled TN-T (●) in the complex of TN-C–TN-T as a function of pCa. Protein concentrations are 0.09 mg/ml of TN-C^{DNS} and 0.23 mg/ml of TN-T in the TN-C^{DNS}–TN-T complex and 0.07 mg/ml of TN-C and 0.07 mg/ml of TN-T^{DNS} in the TN-C–TN-T^{DNS} complex. Solvent condition: 0.3 M KCl–30 mM sodium cacodylate (pH 6.9)–0.9 mM EGTA–varying amounts of $CaCl_2$. The fluorescence intensity is expressed as relative fluorescence intensity with SD ±0.01.

To see whether the Ca^{2+} induced change in TN-C can be propagated to other subunits of troponin, we examined the fluorescence intensity of TN-C–TN-T^{DNS} and TN-C–TN-I^{DNS} as a function of pCa (see Figures 9 and 10). TN-T^{DNS} and TN-I^{DNS} become Ca^{2+} sensitive in the presence of TN-C. In TN-C–TN-T^{DNS} as well as TN-C–TN-I^{DNS}, there is only one transition point at about pCa 5.7, a transition point that is clearly different from the transition point of TN-C^{DNS}. This result indicates that the Ca^{2+} induced changes in the fluorescence intensity of TN-T^{DNS} and TN-I^{DNS} are due to changes in TN-T and TN-I. Therefore we conclude that the Ca^{2+} induced structural change in TN-C is propagated to TN-T as well as to TN-I.

(2) *Effect of Mg^{2+}.* Because in living muscle a few millimoles of Mg^{2+} is always present and because Mg^{2+} may influence the Ca^{2+} binding, we examined whether the Ca^{2+} induced

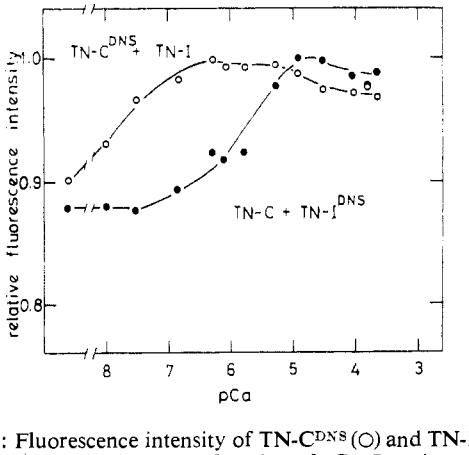

FIGURE 10: Fluorescence intensity of TN-C^{DNS} (○) and TN-I^{DNS} (●) in the complex TN-C–TN-I as a function of pCa. Protein concentrations are 0.1 mg/ml of TN-C^{DNS} and 0.2 mg/ml of TN-I in the complex TN-C^{DNS}–TN-I, 0.1 mg/ml of TN-C and 0.05 mg/ml of TN-I^{DNS} in the complex TN-C–TN-I^{DNS}. The solvent condition is the same as in Figure 9; SD ± 0.01.

TABLE III: Effect of the Presence of Subunits and Combinations of Subunits of Troponin on the DNS Fluorescence of DNS-Labeled Tropomyosin (TM^{DNS}).[a]

	Ca^{2+}	λ Em. max (nm)	I (%)	P
TM^{DNS}	+	518 ± 2	100 ± 2	0.103 ± 0.006
	−	518 ± 2	102 ± 2	0.107 ± 0.003
TM^{DNS}–TN-C	+	503 ± 2	201 ± 2	0.137 ± 0.006
	−	514 ± 2	134 ± 2	0.130 ± 0.005
TM^{DNS}–TN-I	+	512 ± 2	136 ± 2	0.111 ± 0.003
	−	513 ± 2	135 ± 2	0.111 ± 0.002
TM^{DNS}–TN-T	+	514 ± 2	155 ± 2	0.203 ± 0.002
	−	516 ± 2	159 ± 2	0.204 ± 0.003
TM^{DNS}–TN-I–TN-C	+	502 ± 2	185 ± 2	0.115 ± 0.003
	−	511 ± 2	148 ± 2	0.114 ± 0.004
TM^{DNS}–TN-T–TN-C	+	511 ± 2	169 ± 2	0.200 ± 0.005
	−	514 ± 2	162 ± 2	0.203 ± 0.003
TM^{DNS}–TN-T–TN-I–TN-C	+	510 ± 2	182 ± 2	0.188 ± 0.004
	−	513 ± 2	183 ± 2	0.188 ± 0.002

[a] Protein concentrations: 0.24 mg/ml of TM^{DNS}, 0.09 mg/ml of TN-C, 0.13 mg/ml of TN-I, 0.21 mg/ml of TN-T. The protein concentrations of the mixtures are the sums of the protein concentrations of individual components. The fluorescence intensities are expressed as a percentage of the TM^{DNS} fluorescence intensity in the presence of Ca^{2+}. Solvent condition: 0.3 M KCl–30 mM sodium cacodylate buffer (pH 6.9). EGTA (0.9 mM) is present in the minus-Ca^{2+} state and 0.9 mM EGTA–1.3 mM $CaCl_2$ in the plus-Ca^{2+} state.

changes in TN-T and TN-I *via* TN-C can also be induced by Mg^{2+}. As our first experiments suggested that the Ca^{2+} induced change in TN-C is transmitted only to TN-T, we examined the effect of Mg^{2+} on the propagation of the structural change to TN-T and we found that this pathway is not influenced by Mg^{2+} (Kawasaki and van Eerd, 1972). In this paper we present evidence that the propagation of the structural change to TN-I is also Ca^{2+} specific and is not influenced by Mg^{2+}. In Figure 11 is shown the DNS fluorescence intensity of TN-C–TN-I^{DNS}. First EGTA is added, so the fluorescence intensity drops and TN-I becomes the minus-Ca^{2+} state. Further addition of EDTA to remove contaminating Mg^{2+} induces no change the fluorescence intensity. Also upon subsequent addition of $MgCl_2$ no change in fluorescence intensity can be observed. When we finally add $CaCl_2$ the fluorescence intensity comes back to the plus-Ca^{2+} level. Therefore we conclude that the propagation of the Ca^{2+} induced structural change in TN-C to TN-T and TN-I is Ca^{2+} specific and not influenced by Mg^{2+}.

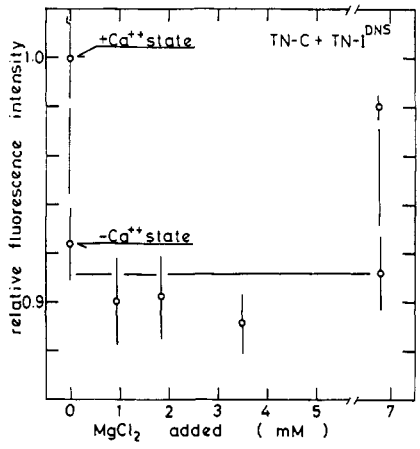

FIGURE 11: Fluorescence intensity of TN-I^{DNS} in the complex TN-C–TN-I^{DNS} as a function of $MgCl_2$ concentration. Protein concentrations are 0.1 mg/ml of TN-C and 0.05 mg/ml of TN-I^{DNS}. Solvent condition: 0.1 M KCl–30 mM sodium cacodylate (pH 6.9). The initial decrease and final increase in fluorescence intensity are due to the addition of 1.8 mM EGTA and 1 mM EDTA and 2 mM $CaCl_2$, respectively. Vertical bars represent standard deviations.

Fluorescence of DNS-Labeled Tropomyosin in Combinations with Subunits of Troponin. Table III and Figure 12 show the effect of the subunits of troponin on the fluorescence properties of DNS-labeled tropomyosin (TM^{DNS}).

TM^{DNS}–TN-C. In Table III we see a large increase in the fluorescence intensity of DNS-labeled tropomyosin and a decrease of the maximum emission wavelength upon addition of TN-C indicating an interaction between tropomyosin and TN-C. Furthermore this interaction is Ca^{2+} sensitive. This

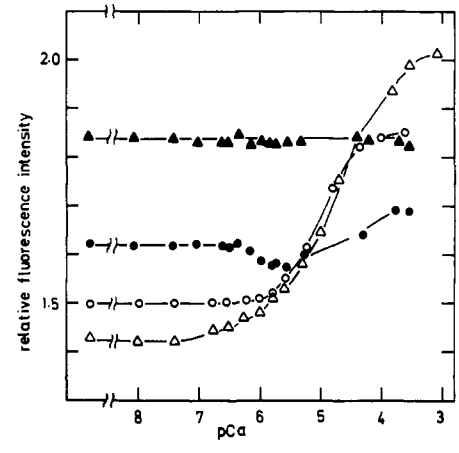

FIGURE 12: Fluorescence intensity of TM^{DNS} as a function of pCa in the complex TM^{DNS}–TN-C (△), TM^{DNS}–TN-I–TN-C (○), TM^{DNS}–TN-T–TN-C (●), and TM^{DNS}–reconstituted troponin (▲). Protein concentrations are 0.4 mg/ml of TM^{DNS}, 0.1 mg/ml of TN-C, 0.17 mg/ml of TN-I, and 0.30 mg/ml of TN-T. Protein concentrations in the mixtures are the sums of the protein concentrations of individual components. Solvent condition is identical with that of Figure 9. The fluorescence intensities are expressed in relative fluorescence intensities with the fluorescence intensity of free TM^{DNS} being 1.00 with SD ± 0.02.

is not expected from gel electrophoresis experiments which showed no binding between tropomyosin and TN-C both in the presence and absence of Ca^{2+}. We subsequently observed the effect of pCa on the fluorescence intensity of the DNS residues in the mixture of TM^{DNS}–TN-C (Figure 12 (Δ)). There is a large increase in fluorescence intensity between pCa 6 and pCa 3. The increase is really due to the presence of TN-C because TM^{DNS} itself does not show a change in fluorescence intensity in the observed range between pCa 9 and pCa 3.

TM^{DNS}–TN-I. Table III shows that the fluorescence of DNS-labeled tropomyosin increases upon addition of TN-I. Also a shift in maximum emission wavelength is observed. These results indicate some interaction between TM and TN-I. Electrophoresis experiments do not show whether TN-I binds to tropomyosin. The inhibition of the ATPase activity of actomyosin by TN-I is greatly enhanced by the presence of tropomyosin (Wilkinson *et al.*, 1971). Therefore the increase in the fluorescence intensity of TM^{DNS} is probably due to binding between TN-I and tropomyosin.

TM^{DNS}–TN-T. The fluorescence intensity of TM^{DNS} increases upon addition of TN-T. Also a large change in the degree of fluorescence polarization and a change in the maximum emission wavelength is observed (Table III). The observed changes are so large that they can be explained by a strong direct binding between TN-T and TM^{DNS}.

TM^{DNS}–TN-I–TN-C. When we look at Figure 12 (○) we see a large change in the fluorescence intensity of TM^{DNS} between pCa 6 and pCa 4. The range of the change observed coincides largely with the change observed in TM^{DNS}–TN-C although its magnitude is reduced (see Figure 12 (Δ)).

TM^{DNS}–TN-T–TN-C. In Table III we see that the addition of TN-C to TM^{DNS} + TN-T has little influence on the DNS fluorescence intensity and the degree of fluorescence polarization. Also in Figure 12 (●) we see that there is only a small change in the TM^{DNS} fluorescence intensity as a function of pCa. The data in Table III and Figure 12 indicate that TN-T induces a new state in tropomyosin which is little affected by the presence of TN-C independent of pCa.

TM^{DNS}–Reconstituted Troponin. Table III shows that the addition of TN-I to the TM^{DNS}–TN-T–TN-C system induces a decrease in the degree of fluorescence polarization and an increase in the fluorescence intensity. The fluorescence intensity of the complete troponin–tropomyosin system in the minus-Ca^{2+} state is higher than that of the TM^{DNS}–TN-T–TN-C system as well as the TM^{DNS}–TN-I–TN-C system. Therefore we conclude that TN-I, as well as TN-T, binds to tropomyosin in the minus Ca^{2+} state of the complete troponin–tropomyosin system. However, the fluorescence intensity of TM^{DNS} does not exhibit any Ca^{2+} sensitivity (Figure 12 (▲)).

Discussion

Ca^{2+} Sensitivity of Individual Subunits. The DNS fluorescence intensity of $TN\text{-}C^{DNS}$ shows an increase followed by a decrease when the Ca^{2+} concentration is increased (Figure 8). When we compare this result with the intrinsic tyrosine fluorescence intensity of TN-C as a function of pCa (Kawasaki and van Eerd, 1972), we see that the tyrosine fluorescence intensity can detect only the change when the high affinity Ca^{2+} binding site is being charged with Ca^{2+}, and not the second lower affinity binding site.

According to our gel electrophoresis results, only TN-I is Ca^{2+} sensitive. However, we would not like to emphasize this because of the unstable nature of TN-I in the absence of TN-C. TN-I sometimes precipitates at low ionic strength without any apparent reason. Furthermore, we could not confirm a Ca^{2+} sensitivity by fluorescence measurements of the intrinsic tryptophan fluorescence, and the DNS fluorescence of $TN\text{-}I^{DNS}$. It seems that only TN-C exhibits Ca^{2+} sensitivity.

Interactions between Subunits. The interactions between TN-C and TN-T as well as TN-I are Ca^{2+} sensitive. According to gel electrophoresis (Figure 6), it seems that the interaction between TN-C and TN-T, and also between TN-C and TN-I, is weaker in the absence of Ca^{2+}. Fluorescence measurements indicate a lower fluorescence intensity of $TN\text{-}T^{DNS}$ and $TN\text{-}I^{DNS}$ in the absence of Ca^{2+} (Figures 9 and 10). Therefore a weakening of the interaction seems to correlate with a decrease in fluorescence intensity. DNS fluorescence measurements show that the structural change in TN-T as well as TN-I occurs at a higher Ca^{2+} concentration than the one where the change in $TN\text{-}C^{DNS}$ occurs due to the loading of the high affinity Ca^{2+} binding sites of TN-C.

How can we explain that a higher concentration of Ca^{2+} is needed to induce a structural change in TN-T and TN-I *via* TN-C than in TN-C itself? TN-C has two kinds of binding sites (Hartshorne and Pyun, 1971) and the total number of Ca^{2+} ions that can be bound by troponin is likely to be 4 or 5 (Bremel and Weber, 1972). If we assume that the high affinity binding site of TN-C can bind more than one Ca^{2+} and if we assume that this binding site must be fully occupied by Ca^{2+} before the Ca^{2+} induced change in TN-C can be propagated to other components, then we would expect the change in TN-T and TN-I to occur at a higher Ca^{2+} concentration. As we can see in Figures 9 and 10, the change observed in TN-T and TN-I is not completed when the high affinity binding site of TN-C is completely loaded with Ca^{2+}. Presumably the loading of the low affinity binding site of TN-C can induce a further change in both TN-T and TN-I. Therefore we think that the change observed in TN-T and TN-I is partly due to the full loading of the high affinity binding site of TN-C and partly due to further loading of the low affinity Ca^{2+} binding site of TN-C.

Muscular contraction is regulated at around pCa 6. Therefore we think that only the change induced in TN-T and TN-I by the loading of the high affinity binding site in TN-C is physiologically important.

To summarize, TN-C interacts with TN-T as well as TN-I, and these interactions are Ca^{2+} sensitive. Most probably, there is no direct interaction between TN-T and TN-I.

Interaction of the Subunits of Troponin with Tropomyosin. Fluorescence measurements (Table III) indicate an interaction of all three individual subunits of troponin with tropomyosin. The interaction between TN-C and tropomyosin is Ca^{2+} sensitive (Figure 12). When we compare Figure 12 with Figure 8 we see that the increase in the fluorescence intensity of TM^{DNS} largely coincides with the binding of Ca^{2+} to the low affinity binding site of TN-C. Electrophoresis experiments indicate no binding between TN-C and tropomyosin at least under the experimental condition used for electrophoresis (low ionic strength and pCa about 5 in the plus Ca^{2+} state). We suppose that the observed increase in the fluorescence intensity of the DNS residues labeled to tropomyosin is due to a nonspecific interaction of TN-C to tropomyosin. This is likely because TN-C has a high negative charge with about one-third of its amino acids being either glutamic acid or aspartic acid (Hartshorne and Pyun, 1971). The net charge of the TN-C molecule changes when the low affinity Ca^{2+} binding site of TN-C is being occupied by Ca^{2+}, and this pre-

sumably is reflected in the change in fluorescence intensity of DNS-labeled tropomyosin. Because the Ca^{2+} induced change is out of the physiologically important range, we do not take it as essential for the regulation of muscular contraction.

TM–TN-I–TN-C. Electrophoresis experiments (Figure 7) show no binding between the complex of TN-C–TN-I in the plus-Ca^{2+} form and tropomyosin. Table III shows that when TN-I is added to the complex of tropomyosin and TN-C in the minus-Ca^{2+} state, the TM^{DNS} fluorescence intensity increases only slightly. There is even a decrease in the plus-Ca^{2+} state. When the interaction between TN-I and tropomyosin is not influenced by TN-C we should expect an increase in the TM^{DNS} fluorescence intensity of about 35% upon addition of TN-I to tropomyosin + TN-C (Table III). It can be seen in Figure 12 that the change in fluorescence intensity in the TM^{DNS}–TN-I–TN-C system largely coincides with the change observed in the TM^{DNS}–TN-C system. Therefore we conclude that the change in the fluorescence intensity of TM^{DNS} in the TM^{DNS}–TN-I–TN-C system is due to TN-C and is only slightly influenced by TN-I. This indicates that the interaction between TN-I and tropomyosin in the presence of TN-C is weaker than in the absence of TN-C. This agrees with the observation that the inhibition of the superprecipitation of actomyosin is released by TN-C even in the absence of Ca^{2+} (Ebashi *et al.*, 1972).

TM–TN-T–TN-C. When we look at the electrophoresis results (Figure 7) we see that the complex TN-T–TN-C binds to tropomyosin independent of the Ca^{2+} concentration. Fluorescence data show that the state of tropomyosin in the complex with TN-T is little influenced by TN-C (Table III), although a slight decrease in fluorescence intensity between pCa 6.5 and pCa 5.5 and a subsequent increase between pCa 5.5 and pCa 4 are observed.

TM–Reconstituted Troponin. This system does not show any Ca^{2+} sensitivity as shown in Figure 12. How can we explain this apparent lack of Ca^{2+} sensitivity? We suppose that the state of tropomyosin in the troponin–tropomyosin system in the minus-Ca^{2+} state is different from the plus-Ca^{2+} state, because without tropomyosin there is no regulation of muscular contraction. Yet we do not detect a change of the fluorescence intensity of TM^{DNS} *vs.* pCa. Therefore, we conclude that at least two effects must play a role. One effect decreases the TM^{DNS} fluorescence intensity at low pCa probably by a weakening of the bond with tropomyosin. The other effect increases the TM^{DNS} fluorescence intensity at low pCa, most likely the direct interaction of TN-C to tropomyosin upon dissociation or weakening of one of the other subunits of troponin. Because the complete troponin–tropomyosin system can regulate the interaction between actin and myosin, the most likely component to dissociate or weaken is TN-I. This is supported by electrophoresis data (Figure 7) indicating no binding of TN-I–TN-C to tropomyosin in the presence of Ca^{2+}. Therefore we conclude that the apparent lack of Ca^{2+} sensitivity of the troponin–tropomyosin system as observed by the TM^{DNS} fluorescence intensity is a combination of a weakening of the bond between tropomyosin and TN-I and a simultaneous strengthening of the interaction between TN-C and tropomyosin when the Ca^{2+} concentration is increased.

Model of Troponin–Tropomyosin Organization. How can we fit these data in a model of the troponin–tropomyosin system? In Figure 13, we have presented a scheme. It is based on the basic assumption that the interaction between actin and myosin is regulated by the state of tropomyosin. We assume that there is only a nonspecific interaction between TN-C and tropomyosin (Figures 7 and 12). This interaction

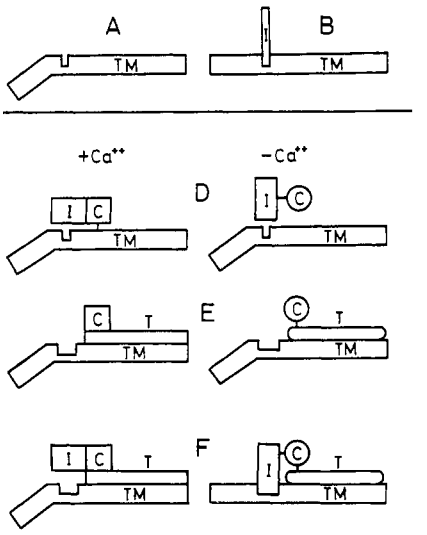

FIGURE 13: Model of the organization of the troponin–tropomyosin system. I stands for TN-I, T stands for TN-T, and C stands for TN-C. Situation A shows free tropomyosin. Situation B shows the interaction between TN-I and tropomyosin. D depicts the TM–TN-I–TN-C system in the presence and absence of Ca^{2+}. E shows the TM–TN-T–TN-C system in the presence and absence of Ca^{2+}. F shows the complete troponin–tropomyosin system in the plus-Ca^{2+} state as well as in the minus-Ca^{2+} state.

is weakened in the presence of either TN-I or TN-T (Figure 12). Also we assume no direct interaction between TN-T and TN-I (Table II, Figure 6). We further assume no Ca^{2+} sensitivity of TN-I in the absence of TN-C. In the model, free tropomyosin is supposed to have a binding site for TN-I as shown by a groove in A. Binding of TN-I to tropomyosin induces a change in tropomyosin (Table III) as shown in B. This state of tropomyosin corresponds to the state which inhibits the interaction between actin and myosin. TN-C, when added to tropomyosin + TN-I, changes the state of TN-I so that TN-I no longer can bind to tropomyosin (Figures 7 and 12). Also the presence of TN-C makes the state of TN-I Ca^{2+} sensitive (Figure 10) but neither the plus-Ca^{2+} state (Figure 7) nor the minus-Ca^{2+} state of TN-I (Figure 12) can bind to tropomyosin (Figure 13D). When TN-T is added (Figure 13E) instead of TN-I to the TM–TN-C system, TN-T induces a change in the state of tropomyosin (Table III, Figure 12) as shown by a widening of the groove in tropomyosin. TN-C induces a change in TN-T which is Ca^{2+} dependent (Figures 6 and 9) but presumably this change does not have a profound effect on the state of tropomyosin (Figure 12) although it might enhance the effect of the Ca^{2+} dependent interaction between TN-I and tropomyosin. The whole reconstituted troponin–tropomyosin system is depicted in Figure 13F. Because of the change in tropomyosin due to the presence of TN-T, TN-I in the minus-Ca^{2+} state can bind to tropomyosin but not in the plus-Ca^{2+} state. The state of TN-C depends on the Ca^{2+} concentration (Figure 8). A change in the state of TN-C induces a change in TN-I as well as in TN-T (Figures 9 and 10). The bonds between TN-C and TN-I and also between TN-C and TN-T are stronger in the plus-Ca^{2+} state (Figure 6). In the plus-Ca^{2+} state TN-I can no longer interact with tropomyosin resulting in the release of the inhibition of the interaction between actin and myosin.

To summarize, the troponin–tropomyosin system inhibits the interaction between actin and myosin when TN-I interacts with tropomyosin. This interaction is regulated by TN-C as well as TN-T. TN-T changes the state of tropomyosin, making

it easier for TN-I to interact with tropomyosin. TN-C changes the state of TN-I. This change is Ca^{2+} sensitive making it possible for TN-I to interact with tropomyosin in the minus-Ca^{2+} state only.

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Chemical Modification of Transfer Ribonucleic Acid Species. Thallium(III)-Mediated Iodination of Yeast Formylatable Methionine Transfer Ribonucleic Acid†

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ABSTRACT: When yeast $tRNA_f^{Met}$ was treated with $TlCl_3$ and NaI in acetate buffer containing $MgCl_2$, 3.2 ± 0.2 mol of iodine was incorporated/mol of tRNA. The stably incorporated iodine was primarily localized in two of the oligonucleotides obtained by T1 ribonuclease digestion of the iodinated $tRNA_f^{Met}$, those containing the amino acid acceptor end and the anticodon sequence of the molecule. In contrast, previously digested tRNA molecules incorporated ^{125}I into seven additional peaks, including the sequence CpGp, which is unmodified in native $tRNA_f^{Met}$ and is located in the double-stranded regions exclusively. The $tRNA_f^{Met}$ iodinated by this procedure accepted amino acid to the same extent as native $tRNA_f^{Met}$, but with a higher K_m and lower V_{max}.

The crystallographic analysis of tRNA demands the introduction of heavy atoms into specific sites in the tRNA crystal. Several approaches may be utilized for this search, either soaking crystals in solutions containing heavy atom ions or organometallic compounds (Schevitz *et al.*, 1972; Kim *et al.*, 1972), treating the α-amino moiety of aminoacyl-tRNA with heavy-atom containing compounds (Schmidt *et al.*, 1972a), treating sites in the native tRNA molecule with heavy atoms (Omilianowski, 1971), or introducing such sites chemically (Hecht and Bock, 1971) or enzymatically (Schlimme *et al.*, 1970). Enzymatic introduction of 5-iodo-CMP into yeast $tRNA^{Phe}$ has been reported (Sprinzl *et al.*, 1972). In addition, Faulkner and Uziel (1971) added iodine to *Escherichia coli* $tRNA^{Phe}$, $tRNA^{Val}$, and $tRNA^{Tyr}$ using I_3^-. Iodine addition was localized at the 2-methylthio-N^6-(Δ^2)-isopentenyl-

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