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Interaction of Rabbit Skeletal Muscle Troponin T and F-Actin at Physiological Ionic Strength†

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ABSTRACT: Troponin T has been shown to interact significantly with F-actin at 150 mM KCl by using an F-actin pelleting assay and ^{125}I-labeled proteins. While troponin T fragment T1 (residues 1–158) fails to pellet with F-actin, fragment T2 (residues 159–259) mimics the binding properties of the intact molecule. The weak competition of T2 binding to F-actin, shown by subfragments of T2, indicates that the interaction site(s) encompass(es) an extensive segment of troponin T. The extent of pelleting of troponin T (or T2) with F-actin is only marginally altered in the binary complex troponin IT (or T2), indicating that the direct interactions either of troponin T (or T2) or of troponin I, or both, with F-actin are weakened when these components are incorporated into a binary complex. The binding of troponin T (or T2) is moderately ($-Ca^{2+}$) or more extensively reduced ($+Ca^{2+}$) in the presence of troponin C. The pelleting of Tn-T seen in the presence of Tn-C ($-Ca^{2+}$) and Tn-I was further reduced when either Tn-I or Tn-C ($-Ca^{2+}$) was added, respectively, to form a fully reconstituted Tn complex. As noted by others, whole troponin shows little sensitivity to Ca^{2+} in its binding to F-actin (–tropomyosin). These and other observations, taken together with the restoration of troponin IC ($\pm Ca^{2+}$) binding to F-actin by troponin T, implicate a role for the interaction of troponin T and F-actin in the thin filament assembly.

The regulation of striated muscle contraction and relaxation is largely controlled through the effects of calcium concentration on the interactions of the thin filament proteins F-actin, tropomyosin (TM),[1] and the three members of the troponin complex, troponins C, I, and T (Tn-C, Tn-I, and Tn-T, respectively). Numerous investigations have demonstrated a multiplicity of interactions between the various components and the modulation of the strengths of these interactions by the binding of Ca^{2+} to Tn-C [for recent reviews and a summary of present knowledge, see Leavis and Gergely (1984), Heeley et al. (1987), and Kay et al. (1987)]. Pertinent to the present investigation and to the molecular mechanism by which this control is exerted are the structural and interactive properties of Tn-T. This protein, now believed to be a highly asymmetric molecule, has been shown to bind to TM through two regions of interaction separated by a distance of 15–20 nm on the thin filament assembly (Ohtsuki, 1975, 1979; Mak & Smillie, 1981; Pato et al., 1981; Pearlstone & Smillie, 1981, 1982, 1983; Flicker et al., 1982; Byers & Kay, 1983; Brisson et al., 1986; White et al., 1987). One of these attachment sites involves an interaction between the T2 region (residues 159–259) of Tn-T and a segment of TM in the central region of its two-stranded coil-coil structure (Ohtsuki, 1975, 1979; Stewart & McLachlan, 1976; Chong & Hodges, 1981; Morris & Lehrer, 1984). This interaction of fragment T2 is sensitive to Ca^{2+} in the presence of Tn-C and -I, to which it also binds (Pearlstone & Smillie, 1978, 1980; Katamaya, 1979; Ohtsuki, 1979). The other attachment site, insensitive to Ca^{2+} except perhaps indirectly, is comprised of the NH_2-terminal portion of Tn-T (fragment T1; residues 1–158) and the head-to-tail overlap region of contiguous TM molecules (Mak et al., 1981; Pato et al., 1981; Pearlstone & Smillie, 1981, 1982, 1983; Brisson et al., 1986; Heeley et al., 1987; White et al., 1987). The interaction of Tn-T with TM at these loci, together with the additional connections of Tn-T with Tn-I and -C through its T2 region as well as the direct binding of Tn-I to F-actin and TM (Potter & Gergely, 1974; Hitchcock, 1975; Pearlstone & Smillie, 1983), is believed to be responsible for the fixing of the troponin complex on the thin filament assembly.

Attempts to demonstrate the existence of an additional link between troponin and the thin filament, via troponin T and F-actin, have been hampered by the poor solubility properties of Tn-T at normal physiological ionic strengths. Working at high ionic strengths (400 mM KCl) to overcome this problem, and using an F-actin pelleting assay, Potter and Gergely (1974) concluded that Tn-T interacted only weakly with F-actin if at all. In another investigation, Johnson and Stockmal (1980) concluded that Tn-T interacted directly with F-actin. This

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[1] Abbreviations: TM, tropomyosin; Tn-C, troponin C; Tn-I, troponin I; Tn-T, troponin T; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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was based on the induction of binding of TM to a chemically modified form of F-actin, which in the absence of Tn-T failed to bind TM. However, other interpretations are possible since Tn-T, now known to span the COOH- and NH_2-terminal overlap regions of contiguous TM molecules (Brisson et al., 1986; White et al., 1987), would be expected to potentiate TM binding to F-actin through its known enhancement of head-to-tail polymerization (Ebashi & Kodama, 1965; Yamamoto & Maruyama, 1973; Jackson et al., 1975). Recently (Heeley et al., 1987), we have observed that, under conditions of low ionic strength, the T2 fragment (residues 159–259) of troponin T has an inductive effect on the binding of nonpolymerizable tropomyosin to F-actin. Since this modified TM lacks the COOH-terminal 11 amino acid residues of intact TM and thus the ability to polymerize head-to-tail, and since fragment T2 is known to interact with the central region of the TM molecule, this inductive effect of fragment T2 on nonpolymerizable TM binding to F-actin may be attributable to its direct interaction with both TM and F-actin. To reexamine this possibility more directly, we have in the present work investigated the binding of radiolabeled Tn-T and its two fragments T1 and T2 to F-actin in the absence of TM at physiological salt concentrations. Using the pelleting assay, we observe a significant interaction between these two proteins and have examined its sensitivity to Ca^{2+} in the presence of Tn-I and -C. We also conclude that the F-actin binding properties of Tn-T are confined to its COOH-terminal region represented by fragment T2 (residues 159–259).

Materials and Methods

Protein Preparations. Actin (Spudich & Watt, 1971), Tn-C, Tn-I, and Tn-T (Mak et al., 1983), and fragments of Tn-T (Pearlstone et al., 1977; Pearlstone & Smillie, 1981) were prepared from rabbit skeletal muscle as described. Their purity was assessed by SDS–polyacrylamide gel electrophoresis (when permissible), amino acid analysis, and NH_2-terminal analysis (Hartley, 1970).

Binding of Tn-T and Its Fragments to F-Actin. F-Actin binding studies were performed as described by Eaton et al. (1975) except for the use of an Airfuge. Iodination of Tn-T and its chymotryptic fragments T1 and T2 with ^{125}I and lactoperoxidase was carried out in 0.4 M KCl and 0.05 M phosphate, pH 7.0, at protein concentrations of approximately 2 mg/mL (T1 and T2) and 1.0 mg/mL (Tn-T), as outlined (Marchalonis, 1969; Eaton et al., 1975). Stock solutions of Tn-C (70–100 μM), T1 (70–100 μM), T2 (70–100 μM), and the cyanogen bromide fragments of Tn-T [CB4, CB5, and CB6 (300–800 μM)] were made up in binding buffer (3 mM imidazole, 0.15 M KCl, 5.5 mM $MgCl_2$, 0.01% sodium azide, 1 mM dithiothreitol, pH 7.0, and either 0.1 mM $CaCl_2$ or 1 mM EGTA) dialyzed overnight against the buffer and stored at 4 °C. Dialysis of peptides CB4, CB5, and CB6 was carried out in Spectrapore membrane 6 tubing (molecular weight cutoff 1000). Due to the low molecular weight of CB7, solutions of this fragment (800 μM) were not dialyzed before use. Tn-I (20–25 μM) was used immediately after the overnight dialysis to minimize oxidation. Tn-T was made up in binding buffer containing either 0.15 M KCl (6–8 μM) or 0.4 M KCl (33 μM). G-Actin was polymerized by dialysis against the appropriate binding buffer (0.1 mM Ca^{2+} or 1.0 mM EGTA; 0.15 M KCl or 0.4 M KCl) with the inclusion of 1 mM ATP, at 4 °C, for 16 h and then used the same day. All protein solutions, except F-actin, were clarified by centrifugation prior to the binding experiment. Protein concentrations were determined by absorbance and by amino acid analyses of triplicate samples. F-Actin concentrations were calculated from the absorbance at 290 and 320 nm using the relationship as in Johnson and Taylor (1978). Experiments with radiolabeled Tn-T, T1, and T2 were carried out by adding the various components in appropriate amounts to a total volume of 100 μL and then mixing gently with a 200-μL Gilson Pipetman. The stoichiometry of binding was established by withdrawing 2 × 10 μL aliquots for counting, before and after centrifugation at 150000g for 20 min at 22 °C in the Airfuge, using a 25-μL Hamilton syringe. The difference was attributed to the quantity of Tn-T or T2 bound. Control experiments showed that in the absence of F-actin a small proportion of radiolabeled Tn-T and T2 was sedimented. The molar binding ratio of Tn-T or T2 to F-actin, at every cited concentration, was therefore calculated as the difference between the amount of Tn-T or T2 sedimenting in the presence and absence of F-actin. Binding ratios derived from experiments with radiolabeled proteins were confirmed by a pelleting assay involving unlabeled components. Pellets containing cold Tn-T or T2 and F-actin were gently washed with binding buffer, dissolved in Laemmli sample buffer containing 1% sodium dodecyl sulfate, and then subjected to electrophoresis at 35 mA on 15% (w/v) polyacrylamide slab gels (0.75 mm thick) as reported (Laemmli, 1970). Gels were stained in a bath containing 0.15% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) ethanol/10% (v/v) acetic acid and destained in 20% (v/v) ethanol/10% (v/v) acetic acid. When this procedure resulted in the adequate separation of Tn-T and F-actin, the relative proportions of Tn-T or T2, and F-actin, were determined by densitometry using a Joyce Loebl Chromoscan 3 scanning densitometer with a 626-nm filter over the light source. Standard curves were constructed to correct for differences in dye binding between the various components. In the case of the cyanogen bromide fragments CB4, CB5, CB6, and CB7, their relative affinities for F-actin were investigated by using a competitive assay involving a constant concentration of radiolabeled T2 and increasing concentrations of the unlabeled fragment. In control experiments, the amount of T2 pelleted in the absence of F-actin was not altered by the addition of these fragments.

Results

Binding of Tn-T to F-Actin. In control experiments with Tn-T alone, a small percentage of the protein was pelleted as shown in Figure 1. In all subsequent experiments (Figures 2–4), therefore, the amount of pelleted Tn-T (either in the absence or in the presence of Tn-I and/or Tn-C) in the absence of F-actin was subtracted from the amount pelleted in the presence of F-actin. The difference was taken as the amount bound to F-actin either directly or indirectly. The addition of Tn-T alone to F-actin at 150 mM KCl led to a precipitation reaction, seen as an increased opalescence of the solution. Similar observations have been made by others under conditions of low ionic strength (Drabikowski & Nowak, 1973; Fujii et al., 1973; Dabrowska et al., 1973). That this phenomenon involves an interaction of Tn-T and F-actin is obvious since the amount of Tn-T pelleted was 10-fold greater in the presence of F-actin than in its absence over the concentration range examined (Figure 1). At physiological ionic strength, as the concentration of Tn-T was increased (from 0 to 4 μM) the molar ratio of bound Tn-T to actin monomer increased in an almost linear manner and did not plateau at a ratio of 1:7 as is observed with troponin in the presence of TM. Comparable molar binding ratios were derived from the analysis of F-actin pellets containing unlabeled Tn-T (see Materials and Methods). At 2.5 μM Tn-T and 7.0 μM F-actin, the quantities of Tn-T bound per actin monomer for the

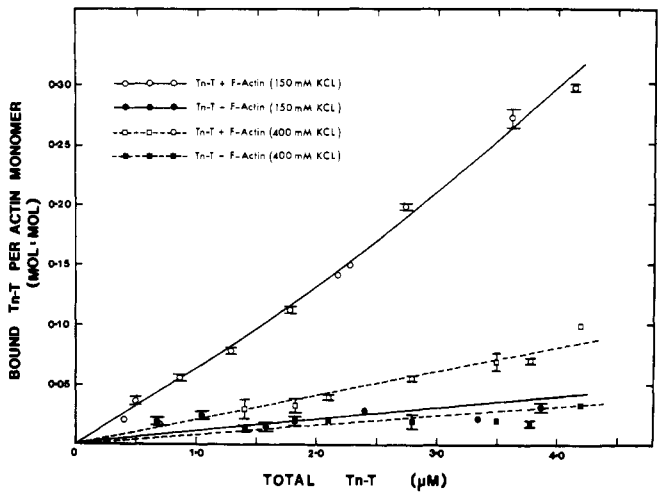

FIGURE 1: Pelleting of rabbit skeletal muscle Tn-T in the presence (○, □) and absence (●, ■) of F-actin, at 150 mM KCl (○, ●) and 400 mM KCl (□, ■). The actin monomer concentration was 7 μM, and the concentration of radiolabeled Tn-T was varied. The buffer conditions were identical in both cases (see Materials and Methods), except that different concentrations of KCl were used. Experimental points are the average of at least two determinations. Where error bars are not shown, identical results were obtained.

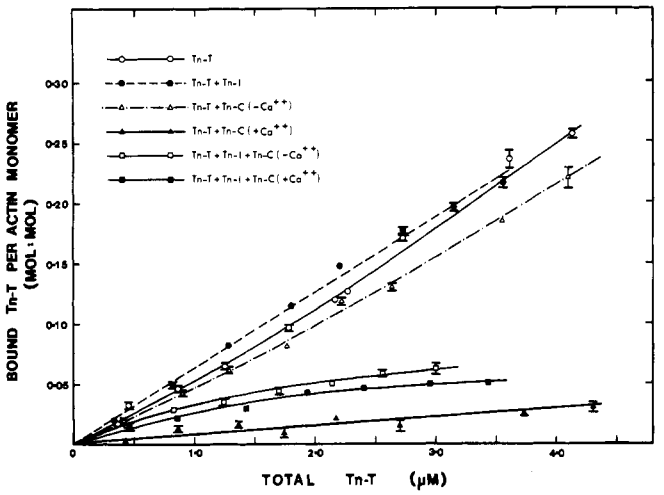

FIGURE 2: Effects of Tn-I, Tn-C (±Ca^{2+}), and Tn-I + Tn-C (±Ca^{2+}) on the sedimentation of Tn-T in the presence of F-actin. The binding curves drawn are the difference between the quantity of radiolabeled Tn-T pelleted in the presence and absence of F-actin. Experimental points for both conditions (±F-actin) were the average of at least two determinations. Where error bars are not shown, identical results were obtained. Buffer conditions (150 mM KCl) were as specified under Materials and Methods. The actin monomer concentration was 7 μM, and the concentration of radiolabeled Tn-T was varied. For mixtures containing more than one component, the respective samples were added in equimolar ratios.

unlabeled and radiolabeled procedures, at 150 mM KCl, were 0.156 (standard deviation = 0.022, n = 4) and 0.148 mol/mol (taken from Figure 1), respectively. The association of Tn-T and F-actin was considerably reduced when similar binding experiments were carried out at 400 mM KCl (Figure 1), confirming the observation of previous workers that the binding at high salt concentrations is weak (Potter & Gergely, 1974). The molar ratios of bound Tn-T to F-actin at 4 μM Tn-T and 7 μM F-actin at the two different ionic strengths were 0.295 (150 mM KCl) and 0.08 (400 mM KCl).

Binding of Tn-T to F-Actin in the Presence of Tn-I and Tn-C (±Ca^{2+}). The data of Figure 2 demonstrate that the pelleting of Tn-T with F-actin is only slightly modified in the presence of Tn-I or Tn-C (−Ca^{2+}). Thus, the binding of the

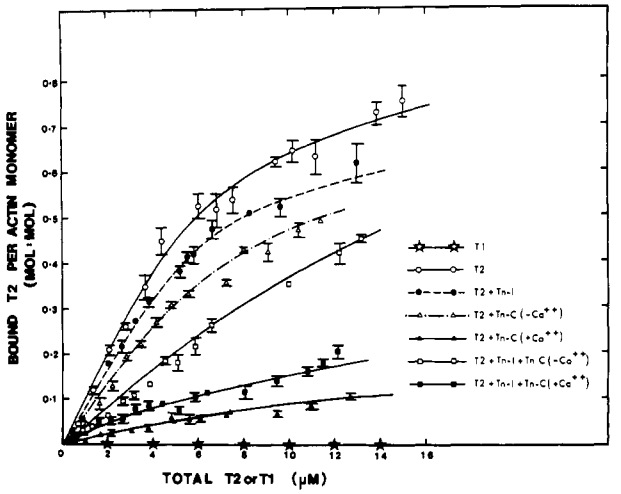

FIGURE 3: Effects of Tn-I, Tn-C (±Ca^{2+}), and Tn-I + Tn-C (±Ca^{2+}) on the sedimentation of fragment T2 and demonstration of a lack of binding of fragment T1 to F-actin. As in Figure 2, the binding profiles represent the difference between the amount of radiolabeled T2 pelleted in the presence and absence of F-actin, where the concentration of F-actin monomer was maintained at 7 μM and the concentration of radiolabeled T1 or T2 was varied. Experimental points for both conditions (±F-actin) were the average of at least two determinations. Where error bars are not shown, identical results were obtained. Where more than one component was tested, the respective samples were added in equimolar ratios. Buffer conditions were the same as in Figure 2.

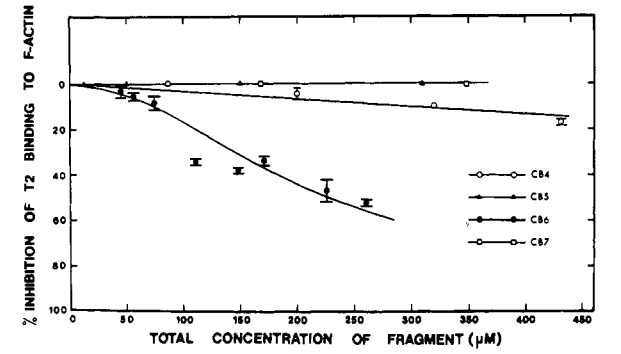

FIGURE 4: Inhibition of binding of fragment T2 to F-actin by other C-terminal fragments of Tn-T. Actin monomer and radiolabeled T2 concentrations were maintained constant at 7 μM and 3 μM, respectively, while the concentration of unlabeled fragment was varied. The amount of T2 pelleted with F-actin in the absence of any other added components was similar to values observed in Figure 3, and made 100%. Buffer conditions were the same as in Figures 2 and 3.

binary complex Tn-TI to F-actin was only slightly greater than for Tn-T alone, and the pelleting of Tn-T in assays containing Tn-C (−Ca^{2+}) was only moderately reduced from that of Tn-T alone. The addition of Ca^{2+} to this latter complex, however, has a more dramatic effect on binding to F-actin, reducing the amount of Tn-T pelleted almost 10-fold. When Tn-I was added to a mixture of Tn-T plus Tn-C (−Ca^{2+}), the amount of Tn-T pelleted was significantly reduced. The addition of Ca^{2+} to this reconstituted Tn complex had only a marginal effect on its binding to F-actin. Thus, as previously observed by others (Potter & Gergely, 1974; Hitchcock, 1975), the binding of whole Tn to F-actin is essentially insensitive to Ca^{2+} concentration.

The precipitation reaction observed when Tn-T alone was added to F-actin was also seen with Tn-T plus Tn-I and with Tn-T plus Tn-C (−Ca^{2+}) but did not occur with the complexes of Tn-T plus Tn-C (+Ca^{2+}) and of Tn-T plus Tn-I plus Tn-C (±Ca^{2+}). Overall, the order of binding was Tn-TI ≥ Tn-T

> Tn-TC ($-Ca^{2+}$) > Tn-TIC ($-Ca^{2+}$) > Tn-TIC ($+Ca^{2+}$) > Tn-TC ($+Ca^{2+}$).

Binding of Tn-T Fragments to F-Actin. Radiolabeled T1 (residues 1–158) and T2 (residues 159–259) were found to differ dramatically in their affinities for F-actin. While fragment T1 showed no interaction, stronger binding was observed with T2 than had been observed with intact Tn-T (Figure 3). At high concentrations of T2 ($\simeq$30 μM), the interaction approached a saturation limit of T2/actin monomer of 0.9:1.0 (moles per mole) (data not shown). The apparent binding constant was calculated to be 0.35×10^6 M^{-1}. Molar binding ratios estimated by densitometry, as outlined under Materials and Methods, were in good agreement with those obtained by using ^{125}I-labeled T2. For example, at 3.0 μM T2 and 7.0 μM F-actin, the ratios of radiolabeled and unlabeled bound T2 to actin monomer were 0.275 (taken from Figure 2) and 0.254 (moles per mole) (standard deviation = 0.039, $n = 4$), respectively. The T2 fragment also mimicked the properties of the parent molecule in precipitating F-actin at 150 mM KCl, and in being pelleted to some extent in the absence of F-actin. This latter effect was corrected for in a manner identical with that described for intact Tn-T.

From the data presented in Figure 3, it is evident that the pelleting of T2, when present in binary complexes with either Tn-I or Tn-C ($\pm Ca^{2+}$), shows only minor differences to what was observed with intact Tn-T and therefore essentially follows a similar pattern. However, a significant discrepancy may be noted between the pelleting of the ternary complexes Tn-T2IC ($-Ca^{2+}$) and Tn-TIC ($-Ca^{2+}$). Thus, while the addition of Tn-I and Tn-C ($-Ca^{2+}$) to T2 led to only a moderate reduction in the extent to which it was pelleted, the pelleting of intact Tn-T was more dramatically reduced by the inclusion of the other two troponin components in the absence of Ca^{2+}. Associated with this difference is the extent to which the two ternary complexes are affected by Ca^{2+}. While the binding of the Tn-T2IC ($-Ca^{2+}$) complex is significantly reduced by the addition of Ca^{2+}, the binding of the reconstituted Tn complex to F-actin is essentially insensitive to Ca^{2+} concentration. Overall, the order of binding was Tn-T2 > Tn-T2I > Tn-T2C ($-Ca^{2+}$) > Tn-T2IC ($-Ca^{2+}$) > Tn-T2IC ($+Ca^{2+}$) > Tn-T2C ($+Ca^{2+}$).

In attempts to more precisely delineate that region of the polypeptide chain responsible for the binding of Tn-T and fragment T2 to F-actin, a number of smaller cyanogen bromide fragments of Tn-T were tested for their ability to compete with T2 in the pelleting assay (Figure 4). Of those tested, CB6 (residues 239–259) was the most effective while CB4 (residues 176–230) showed some degree of competition. The ability of CB6 to interact with F-actin was consistent with the fact that it promoted precipitation of the latter, as was observed with intact Tn-T and fragment T2. Because of the high molar ratios required for effective competition, however, it is clear that CB6 (and CB4) binds much more weakly to F-actin than does intact T2. In similar experiments, the cyanogen bromide peptides CB5 (residues 152–175) and CB7 (residues 231–238) were found to be ineffective in this respect.

Discussion

At our present level of understanding, the molecular architecture of the thin filament is thought to be characterized by at least three attachment points between the Tn complex and the F-actin–TM filament system. The interactions between Tn-I and F-actin–TM filament system. The interactions between Tn-I and F-actin–TM and also the C-terminal portions of Tn-T (residues 159–259) and TM are believed to be Ca^{2+} sensitive, while the N-terminal section of Tn-T (residues 1–158) binds to the head-to-tail overlap region of TM in a manner than is insensitive to Ca^{2+} except perhaps indirectly. In the present work, we have addressed the question of the possibility of an additional linkage involving Tn-T and F-actin. While this question has been considered by others (Potter & Gergely, 1974; Johnson & Stockmal, 1980), previous experiments have been complicated by the poor solubility properties of Tn-T at low ionic strengths (Potter & Gergely, 1974) and the complexities of interpretation of binding data in a multicomponent system (Johnson & Stockmal, 1980). However, using radiolabeled Tn-T and the F-actin pelleting assay in the Airfuge, we have found that adequate concentrations of Tn-T (5–6 μM) can be prepared at 150 mM KCl to examine directly the interaction of these two components. Although in controls some pelleting of Tn-T alone occurred in this assay, presumably due to the tendency of Tn-T to aggregate under nondenaturing conditions (Byers & Kay, 1983), the presence of F-actin increased these amounts by a factor of about 10, thus demonstrating the direct interaction of the two components. Because of the limited solubility of Tn-T, it was not possible to extend the binding data to concentrations greater than 4 μM. However, even at these concentrations, it is clear that the ratio of bound Tn-T to actin monomer exceeds the value of 1:7 observable for whole Tn in the presence of TM. A lower level of binding was observed when the experiment was carried out at 400 mM KCl. This result is in agreement with past studies, where a weak interaction between Tn-T and F-actin was reported at an identical salt concentration. That the interaction of Tn-T and F-actin is markedly dependent on ionic strength and is accompanied by a precipitation of the complex does not necessarily detract from its significance since the solubility or insolubility of a complex is not in itself an indication of the specificity or nonspecificity of that interaction.

When the two chymotryptic fragments T1 and T2 were examined in the assay system, it was found that while T1 showed no evidence for interaction with F-actin, fragment T2 mimicked the binding properties of whole Tn-T including the formation of an insoluble complex with F-actin. Because of the increased solubility properties of fragment T2 compared with those of whole Tn-T, it was possible to extend the binding data to higher molar concentrations of the fragment. These data indicated that the binding of T2 to F-actin was approaching saturation at about 1 mol of T2/mol of actin monomer. It would thus appear that in the intact thin filament assembly, TM is required to correctly order Tn-T on the F-actin structure and thereby restore a stoichiometry of one Tn-T per seven actin monomers.

The binding of fragment T2 but not T1 to F-actin indicates that the F-actin binding properties are associated with the COOH-terminal region of the Tn-T molecule. This was further supported by the abilities of certain subfragments of T2 to compete with T2 for binding to F-actin (Figure 4), of which cyanogen bromide fragment CB6 (residues 239–259) is the most effective. This COOH-terminal segment of Tn-T contains a cluster of 7 positively charged residues, no acidic residues, and a COOH-terminal sequence of 10 residues which is highly conserved in the several Tn-T isoforms whose amino acid sequences have been reported [see Pearlstone et al. (1986) for a summary of these]. However, for several reasons, it is likely that the F-actin binding site on Tn-T is not confined to its extreme COOH-terminal end but rather spans a more extensive region of its polypeptide chain. First, fragment CB4 (residues 176–230) is also seen to reduce the binding of T2 to F-actin (Figure 4). In addition, both CB6 and CB4 appear to have significantly reduced affinities for F-actin since large

molar excesses of these peptides are required for effective competition of the binding of fragment T2 to F-actin. Finally, removal of the last three residues (Arg-Trp-Lys) from the C terminus of Tn-T by digestion with a combination of carboxypeptidases A and B did not significantly alter the F-actin binding properties of a troponin complex reconstituted with the truncated molecule (data not shown). These results are to be expected if the F-actin binding site on Tn-T encompasses part or all of the sequence contained in each of fragments CB4 and CB6. Differences in the conformation of the two small fragments (CB4 and CB6) compared to the larger fragment T2 and the intact protein may also contribute toward a reduction in their affinity for F-actin. In any case, the observations indicate that an extensive region encompassing residues 176–259 of Tn-T is involved in its interaction with F-actin although residues 239–259 may be of particular significance in this respect. Since the T2 region of Tn-T has also been shown to bind to Tn-I and Tn-C (Pearlstone & Smillie, 1978, 1980; Katayama, 1979), it was of considerable interest to assess the effects of the addition of these components on the pelleting of Tn-T and T2 with F-actin. Surprisingly, the inclusion of Tn-I to form a Tn-TI or Tn-T2I binary complex only marginally increased the pelleting of Tn-T and in the case of T2 actually decreased the amount (see Figures 2 and 3). This suggests that the direct interactions either of Tn-T (or T2) or of Tn-I or of both with F-actin are weakened when these components are incorporated into the binary complex. A similar result is also seen with Tn-TC ($-Ca^{2+}$) and Tn-T2C ($-Ca^{2+}$) in which the amount of Tn-T (or T2) pelleted with F-actin is decreased when incorporated into these binary complexes. In this case, the weaker binding can be attributed directly to that involved between Tn-T (or T2) and F-actin since Tn-C is known not to bind to F-actin. The weakening of this interaction between Tn-T (or T2) and F-actin in the presence of Tn-C is much more pronounced when Ca^{2+} is included in the assay mixture.

When both Tn-I and Tn-C ($-Ca^{2+}$) are added to Tn-T or T2 to form the corresponding ternary complexes, the amount of Tn-T or T2 pelleted with F-actin is further reduced relative to that observed in the binary complexes of either Tn-TI (or Tn-T2I) or Tn-TC (or Tn-T2C) in the absence of Ca^{2+}. This further weakening of the direct interaction between Tn-T (or T2) or Tn-I or both and F-actin is more dramatic, however, for the reconstituted Tn complex (Tn-TIC) than for the Tn-T2IC ternary complex. When Ca^{2+} is added to these ternary complexes, the binding of the reconstituted Tn to actin is now only marginally reduced (Figure 2) while that of the Tn-T2IC complex is markedly reduced. This relatively weak binding of whole Tn to F-actin in the absence of TM and its insensitivity to Ca^{2+} are in agreement with previous observations on this system (Potter & Gergely, 1974; Hitchcock, 1975). The aforementioned differences between the ternary complexes of Tn-TIC and Tn-T2IC under plus and minus Ca^{2+} conditions presumably reflect the absence of the NH_2-terminal region of Tn-T (fragment T1; residues 1–159) in the Tn-T2IC complex. Since there is no evidence for the direct interaction of the T1 region with actin, Tn-I, or Tn-C, these differences must indicate that the T1 portion of Tn-T can influence the interaction properties of the T2 region with the other components when it is a constituent part of the whole Tn-T molecule. Thus, while the T1 region of the Tn-T molecules is believed to bind directly only to TM in the complete thin filament assembly, its absence in the Tn-T2IC complex clearly does have an influence on the latter's interaction with F-actin in the absence of TM. At our present level of understanding of the system, these observations can only be interpreted in terms of conformational effects of the T1 region on those of T2 and thus on its interaction properties when both are present as parts of the complete Tn-T molecule.

While it is difficult to assess the relative contribution of the Tn-I and Tn-T components to the binding of whole Tn to F-actin, the following observations are pertinent. First, both Tn-T and Tn-I by themselves bind to F-actin as does the binary complex Tn-IT. While the addition of Tn-C to Tn-T marginally reduces the binding of Tn-T in the absence of Ca^{2+} and more dramatically in its presence, the formation of the Tn-CI complex has been reported (Potter & Gergely, 1974; Hitchcock, 1975) to completely eliminate Tn-I binding both in the absence and in the presence of Ca^{2+}. Further, while the addition of Tn-I to Tn-TC ($-Ca^{2+}$) reduces the binding to actin, the addition of Tn-T to Tn-IC ($\pm Ca^{2+}$) restores a degree of binding in both $\pm Ca^{2+}$. Taken together with other observations including the ability of Tn-T and T2 to induce the binding of nonpolymerizable TM to F-actin (Heeley et al., 1987) as well as the induction of intact TM binding to a modified form of F-actin by Tn-T (Johnson & Stockmal, 1980), these considerations all suggest a direct role for Tn-T binding to F-actin in the thin filament assembly.

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Effect of Poly(ethylene glycol) on the Ca^{2+}-Induced Fusion of Didodecyl Phosphate Vesicles†

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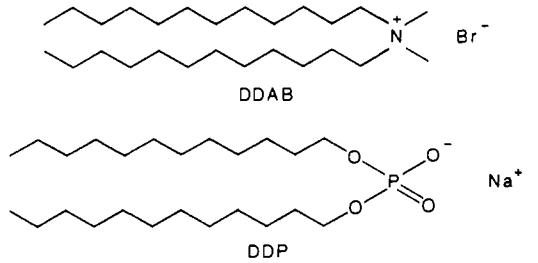
ABSTRACT: This paper reports a study of the effect of the dehydrating agent poly(ethylene glycol) (PEG) on didodecyl phosphate (DDP) bilayers and on the fusion activity of DDP vesicles as a function of the molecular weight of PEG. PEG 8K in a concentration of 10 wt % does not induce fusion. However, Ca^{2+}-induced fusion is promoted as reflected by a lowering of the Ca^{2+} threshold concentration. This effect can most likely be attributed to the dehydrating capacity of the polymer. Interestingly, low concentrations (0.1 wt %) of PEG 20K induce a moderate fusion capacity. At higher concentrations (0.5 wt %) fusion is inhibited, irrespective of the presence of Ca^{2+}. These molecular weight dependent effects can be rationalized by taking into account that the clouding temperature differs for PEGs of different molecular weights. In the case of PEG 20K a microscopic phase separation will occur at the bilayer–water interface because PEG–PEG interactions and presumably PEG–DDP interactions are favored over PEG–water interactions. As a consequence, the DDP vesicle surface becomes covered with PEG 20K, resulting in a steric stabilization of the vesicles. This will impede or prevent, depending on the polymer concentration, the vesicles from approaching each other sufficiently close for fusion to occur.

Ion-induced fusion of bilayer vesicles formed from the synthetic amphiphiles didodecyldimethylammonium bromide (DDAB)[1] and didodecyl phosphate (DDP) is triggered by a



perturbation of the bilayer–water interface (Rupert et al., 1985, 1986, 1987). The stability of the water–bilayer interface, and in a broader sence that of the bilayer itself, is affected by the headgroup structure, counterion binding, and, in particular, by headgroup hydration (Portis et al., 1979; Rand, 1981; Rupert et al., 1987). For fusion to occur, dehydration of the headgroup is required to reduce the strongly repulsive hydration forces, which would otherwise prevent the bilayers from coming into close, i.e., fusion-susceptible, contact (Portis et al., 1979; Rand, 1981; Evans et al., 1986; Wilschut & Hoekstra, 1986; Hoekstra & Wilschut, 1988). Furthermore, headgroup hydration is also of paramount importance for the bilayer-to-hexagonal H_{II} phase transition (Brown et al., 1986; Rupert et al., 1987). It is anticipated, therefore, that manipulation of the hydration of the headgroup of the amphiphiles

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[1] Abbreviations: DDP, didodecyl phosphate; PEG, poly(ethylene glycol); *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; DDAB, didodecyldimethylammonium bromide; PC, phosphatidylcholine; PS, phosphatidylserine; RET, resonance energy transfer; IMI, inverted micellar intermediate; LCST, lower critical solution temperature.

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