Tropomyosin is a coiled-coil protein that polymerizes by head-to-tail interactions in an ionic strength-dependent manner. We produced a recombinant full-length chicken α-tropomyosin containing a 5-hydroxytryptophan residue at position 269 (formerly an alanine), 15 residues from the C terminus, and showed that its fluorescence intensity specifically reports tropomyosin head-to-tail interactions. We used this property to quantitatively study the monomer-polymer equilibrium in tropomyosin and to calculate the equilibrium constant of the head-to-tail interaction as a function of ionic strength. Our results show that the affinity constant changes by almost 2 orders of magnitude over an ionic strength range of 50 mM (between \( I = 0.045 \) and 0.095). We were also able to calculate the average polymer length as a function of concentration and ionic strength, which is an important parameter in the interpretation of binding isotherms of tropomyosin with other thin filament proteins such as actin and troponin.

Skeletal muscle tropomyosin (Tm) is a 284-residue dimeric in-register coiled-coil protein that plays a central role in the regulation of muscle contraction through its interactions with actin and troponin (Tn) in the thin filament (1–5). Analysis of its primary structure revealed the existence of an extensive and almost perfect heptad repeat \( (abcdefg) \) in which hydrophobic residues at positions \( a \) and \( d \) create a dimerization interface that stabilizes the coiled-coil structure (6, 7). Further analysis of tropomyosin sequences also uncovered seven 39.5-residue pseudo-repeats, which may reflect the tropomyosin 7:1 binding stoichiometry with actin (8). Also associated with both tropomyosin and actin is the troponin complex, which consists of the \( \text{Ca}^{2+} \) binding subunit (troponin C), the inhibitory subunit (troponin I), and the tropomyosin binding subunit (TnT). Tn-Tm interactions occur mainly between the C-terminal half of TnT and the region near position 190 of Tm and between the N-terminal half of TnT and the C-terminal of the Tm molecule (for review, see Refs. 9–13).

Muscle tropomyosin high viscosity at low ionic strength was noted from the time of its first isolation (14). Soon thereafter Tsao et al. (15) describe the ionic strength dependence of the viscosity in more detail and Kay and Bailey (16) relate changes in the polymer length with salt concentration and propose that polymerization is due to simple end-to-end-or head-to-tail- aggregation, which was later confirmed by in-depth studies of cardiac Tm via sedimentation velocity, sedimentation equilibrium, osmometry, viscometry, and optical rotatory dispersion (17).

Although muscle Tm is acetylated at its N terminus, recombinant non-fusion Tm (nFTm) expressed in bacteria lacks this modification and does not polymerize or bind to actin (18, 19). Recombinant Tm expressed in insect cells (20) or yeast (21) has its N terminus acetylated and is functional. Because the N-acetylated initiation methionine in the native protein occupies an internal position in the coiled-coil structure (position \( a \)), repulsions between the positively charged \( \alpha \)-amino groups in the recombinant protein were thought to destabilize the local coiled-coil structure as well as the head-to-tail interaction responsible for polymerization (19). An N-terminal dipeptide fusion (ASTm) reduces these repulsions by placing the charged \( \alpha \)-amino group at an external position \( (f) \) and restores the polymerization and actin binding properties of Tm (19). This view is confirmed by the recent high resolution structures of N-terminal fragments of acetylated and non-acetylated tropomyosin; the acetylated fragment maintains the coiled-coil structure up to the N terminus (22), whereas the first two residues in the unacylated fragment are non-helical (23). The structure of full-length Tm has only been resolved to 7 Å due to its high flexibility and asymmetry (24).

Tm crystals show a repeat distance of 410 Å, less than that expected for a molecule containing 284 \( \alpha \)-helical residues (~423 Å). The size difference was proposed to correspond to the overlap between Tm molecules (25, 26). The removal of more than three C-terminal amino acids or chemical modification of the \( \epsilon \)-NH\(_2\) group of Lys-7 abolishes Tm polymerization (27). Heeley et al. (28) show that native Tm, which is phosphorylated at Ser-283, has a greater viscosity than unphosphorylated Tm, but actin and Tn binding ability was the same for both forms. McLachlan and Stewart (29) propose a model for the Tm head-to-tail interaction involving nine N-terminal and C-terminal residues from each tropomyosin chain, in which they attempted to maximize hydrophobic and electrostatic interactions.

Very few quantitative measures of the thermodynamics of the tropomyosin head-to-tail interaction have been performed (30–32) due to the heterogeneity of solutions containing polymers of different lengths. Assai (30) attempts to calculate \( K \) as a function of ionic strength but only in the range 0–12 mM KCl,
2 mM phosphate, whereas Ogi et al. (31) calculates $K$ at 0.1 and 1 M. Sano et al. (32) studies the head-to-tail interaction using recombinant N- and C-terminal tropomyosin fragments. All groups arrived at $K$ values between $2 \times 10^4$ and $1.25 \times 10^5$ M$^{-1}$ for ionic strengths that varied from less than 0.01 to 1.0, which is surprising in light of the strong ionic strength dependence of viscosity of micromolar tropomyosin solutions in this range.

Quantitative measures of the thermodynamics of the Tm head-to-tail interaction are important for the interpretation of binding isotherms between Tm and other thin filament proteins, especially since mutations that influence Tm polymerization also affect its interactions with Tn and actin. Because Tm interactions with these proteins are strongly ionic strength-dependent, a quantitative analysis of the ionic strength dependence of polymerization thermodynamics is also important. With this aim in mind, we have produced a recombinant tropomyosin with a fluorescent 5-hydroxypyrophophan probe at position 269, located 15 residues from the C terminus of the polypeptide chain. 5-Hydroxypyrophophan probes at other positions in this region are specifically sensitive to Tm Tn binding and actin binding (33, 34). We show that the fluorescence of this probe is sensitive to Tm polymerization and use this property to study polymerization thermodynamics as a function of ionic strength.

**EXPERIMENTAL PROCEDURES**

**Construction of Vectors for Bacterial Expression of Tropomyosin Mutants**—The alanine codon at position 269 of the chicken skeletal act-tropomyosin cDNA (35) was substituted for a tryptophan codon by PCR-mediated site-directed mutagenesis using the bacterial expression vector constructs pET-MASTmy and pET-Tmy (19) as templates. These vectors direct the expression of tropomyosin with a dipeptide Ala-Ser at position 269, located 15 residues from the C terminus of the tropomyosin sequence.

**Protein Purification**—Expression and purification of the mutant tropomyosin with 5-hydroxypyrophophan residue were determined using a F-4500 Hitachi spectrofluorimeter (excitation, 312 nm; excitation and emission slit bandwidths, 5 nm; temperature, 25°C). The effect of the ionic strength on the emission spectrum of AS269(50HW) was determined in fluorescence buffer with NaCl concentrations varying from 10 to 350 mM and Tm concentrations varying between 0.5 and 8 μM. Each point of each curve in Fig. 3A corresponds to the fluorescence intensity of a 1.5-ml mixture of the protein at a specific NaCl concentration. Samples were equilibrated for 7 h at 25°C before taking the spectra, and the emission intensity was determined by summing the emissions at all wavelengths between 330 and 380 nm. The fluorescence intensity was corrected for the inner filter effect, which was only significant at high Tm concentrations and never exceeded 14%.

**Calculation of the Affinity Constant of the Tropomyosin Head-to-tail Interaction and Its Dependence on Ionic Strength**—We begin with the premise that the probe can exist in two environments, free or “polymerized,” with different fluorescence intensity in each state ($F_{\min}$ and $F_{\max}$, respectively; see Figs. 1 and 3). The free probe concentration is equal to the polymer concentration, since each polymer has only one C terminus regardless of its length (Fig. 1D). As the ionic strength increases, the affinity of the head-to-tail interaction decreases until essentially all Tm is in the monomeric state, at which point the base line of the titration (see the arrow in Fig. 3A) corresponds to the intrinsic fluorescence of the free probes ($F_{\min}$). The data in Fig. 3A were normalized with respect to the Tm concentration and the $F_{\max}$ for each curve. We define $f_i$ as the fraction of probes in the free environment, i.e., the fraction of Tm monomers whose C termini are not involved in a head-to-tail interaction.

$$f_i = (F_{\max} - F_{\min})/F_{\max} - 1$$

where $F_N$ and $F_{\max}$ are, respectively, the actual and maximal fluorescence intensities, both normalized with respect to protein concentration and to $F_{\min}$. $f_i$ is also equivalent to $<i>^{-1}$, where $<i>$ is the average number of monomers per polymer chain.

$$(\lambda_i + \lambda_2 + \lambda_3 + \lambda_4)/\lambda_{tot} = (\sum c_i K_i^{-1})/\lambda_{tot}$$

where $\lambda_{tot}$ is the total tropomyosin concentration, $\lambda_i$ is the i-mer concentration, and $K_i$ is the affinity constant for the head-to-tail interaction (45, 46).

We can then show the following.

$$f_i = (\sum (1 - K_i^{-1}))/\lambda_{tot}$$

Because $N = x/(1 - x)$, we arrive at the expression,

$$f_i = \lambda_i/\lambda_{tot} (1 - K_i^{-1})$$

so long as $\lambda_i < K_i^{-1}$. In their analysis of linear polymerization equilibria, Oosawa and Kasi (45) show that,

$$\lambda_{tot} = \sum \lambda_i = \lambda_i (1 - K_i^{-1})$$

which can be rearranged to

$$\lambda_i = (2K_{\lambda_i} - 1 - (4K_{\lambda_i} + 1)^{1/2})/(2K_{\lambda_i}^{5/4})$$

Equations 4 and 6 may be combined to express $f_i$ in terms of $K$ and $\lambda_{tot}$ as follows.

$$f_i = (2(4K_{\lambda_i} + 1)^{1/2} - 1)/(1/K_{\lambda_i})$$

Finally, Equations 1 and 7 may be combined to express $F_N$ in terms of $F_{\max}$, $K$, and $\lambda_{tot}$ as follows.

$$F_N = F_{\max} - ((F_{\max} - 1)(2(4K_{\lambda_i} + 1)^{1/2} - 1)/(1/K_{\lambda_i}))$$

Equations 7 and 8 were used to fit the experimental data in Fig. 3B to simultaneously obtain values for $K$ and $F_{\max}$ (at each ionic strength) using the program SigmaPlot 3.0 (Jandel Scientific). Using a fixed value of $F_{\max} = 1.55$ (see Fig. 3A), we calculated $K$ at each ionic strength, the values of which are shown in Fig. 4. The values for $K$ obtained using a fixed or variable value for $F_{\max}$ were not significantly different (>50% and usually much less) for the ionic strengths shown in Fig. 4 (between $I = 0.045$ and 0.095). As can be seen by inspection of Fig. 3, at ionic strengths below or above this range (20–70 mM NaCl), the observed change in intrinsic fluorescence as a function of tropomyosin concentration was too little to accurately estimate $K$. 
RESULTS

We analyzed a series of recombinant Tm mutants containing 5-hydroxytryptophan at a number of positions along its primary structure. The fluorescent probe can be selectively excited at wavelengths above 300 nm, where tryptophan-containing proteins do not absorb (Fig. 1C). We found that the fluorescence of 5-hydroxytryptophan incorporated at position 269 (AS269(5OHW)) is sensitive to ionic strength variations, which lead to Tm depolymerization (Fig. 1A). Although the $\lambda_{\text{max}}$ of the emission spectrum remains constant, the fluorescence intensity decreases $\approx$38% as the NaCl concentration is raised from 10 to 300 mM (Fig. 1A). The fluorescence of the mutant with tryptophan (instead of 5-hydroxytryptophan), incorporated at the same position (AS269W), is also sensitive to ionic strength, but in this case the decrease in intensity is accompanied by a red shift (data not shown). This combined spectral shift plus intensity change complicates quantitative analysis in terms of a two-state equilibrium (47). For this reason, we chose to use the variation of the intensity of fluorescence of AS269(5OHW) to investigate the head-to-tail interaction of Tm. By exciting at 312 nm, we eliminate any fluorescence contribution from the small fraction (38, 48) of Tm molecules containing tryptophan instead of 5-hydroxytryptophan (Fig. 1C).

In contrast to AS269(5OHW), the fluorescence of the non-fusion mutant labeled at position 269 that does not polymerize, nf269(5OHW), is insensitive to ionic strength variations (Fig. 1B). Furthermore, the fluorescence intensities of other polymerizable Tm mutants labeled with 5-hydroxytryptophan at nearby positions in the primary structure (positions 261, 263, and 267 (33)), in the center (position 185), or in the N-terminal half of the protein (positions 90, 101, 111, and 122 (38)) are insensitive to ionic strength variations.

We attempted to determine whether the mutation at position 269, both in the presence and absence of the dipeptide N-terminal fusion, significantly affected other properties of Tm such as actin-binding (+/−Tn), circular dichroism, and viscosity (Fig. 2). No significant differences in the CD spectra of the

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** A and B, fluorescence emission spectra ($\lambda_{\text{ex}}$ = 312 nm) of AS269(5OHW) (A) and nf269(5OHW) (B) at 10 mM and 300 mM NaCl. C, excitation spectra of AS269(5OHW) and nf269(5OHW) with 5-hydroxytryptophan incorporated at position 269 and of AS269W with tryptophan at the same position. The incorporation of 5-hydroxytryptophan in AS269(5OHW) and nf269(5OHW) is apparent by the shoulder in their spectra, which is absent in AS269W. D, a scheme illustrating the polymerization of Tm. The 5-hydroxytryptophan probe can exist in one of two environments, (i) a polymerized environment (filled circles in the figure, maximum fluorescence intensity), where the C-terminal end of the tropomyosin molecule to which the probe is covalently attached interacts with the N-terminal end of another tropomyosin molecule, and (ii) a free environment (open circles, minimum fluorescence intensity), which occurs only at the C-terminal ends of each tropomyosin polymer and in free monomers.

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** A, viscosity assays of ASTm and nTm with and without the 5-hydroxytryptophan probe at position 269. Conditions: 8 μM Tm in 10 mM imidazole, pH 7.0, and 2 mM DTT. X and □ refer to ASTm and AS269 (5OHW), respectively, in 300 mM NaCl. B, co-sedimentation assays to detect the actin binding ability of AS269(5OHW) and nf269(5OHW) in the absence and in the presence of Tn. Mixtures (M) before and supernatants (S) and pellets (P) after ultracentrifugation were analyzed by SDS-PAGE. Conditions: 2 μM Tm, 14 μM actin, and 1 mM DTT. Identical results were observed when the NaCl concentration was raised to 150 mM. C, CD spectra of ASTm and nTm with and without the 5-hydroxytryptophan probe at position 269. Conditions: 10 μM Tm in 50 mM NaH$_2$PO$_4$, pH 7.0, 0.5 mM DTT, 0.5 mM EDTA, and 100 mM KCl. Except for CD experiments, all samples were pre-equilibrated for 7 h at 25 °C.
four proteins (ASTm, nfTm, AS269(5OHW), and nf269(5OHW)) were observed (Fig. 2C), indicating that the mutations do not significantly affect the overall secondary structure of the molecule. Local secondary structure changes induced by the mutation, however, cannot be ruled out due to limitations in the sensitivity of this method. Because residue 269 corresponds to an external position (c) in the heptad repeat of tropomyosin (29), the stereospecific interactions at the interface between the two α-helices in the coiled-coil structure should not be disrupted.

The kinematic viscosity of the four recombinant tropomyosins was determined at a series ofionic strengths (Fig. 2A). As expected (19), recombinant tropomyosins with an N-terminal Ala-Ser dipeptide fusion present salt-dependent viscosity dependence, whereas non-fusion recombinant tropomyosins have significantly reduced viscosity at all ionic strengths tested. We did not observe any significant difference between the viscosities of ASTm and AS269(5OHW) or between nfTm and nf269(5OHW) (Fig. 2A). This indicates that the mutation at position 269 does not lead to significant changes in the intermolecular interactions responsible for tropomyosin polymerization.

We checked the actin and troponin binding ability of the four tropomyosins in co-sedimentation assays (Fig. 2B and data not shown). These assays showed that AS269(5OHW) is able to bind actin and mediates the binding of troponin to thin filaments, similar to ASTm (19). Furthermore, nf269(5OHW) does not bind actin on its own but does bind in the presence of troponin, just as previously observed for nfTm (Fig. 2B and Ref. 19).

The above analysis indicates that the probe at position 269 does not significantly affect tropomyosin interactions with actin or troponin or its salt-dependent polymerization. Because the only chemical difference between AS269(5OHW) and nf269(5OHW) is the presence or absence of an N-terminal dipeptide Ala-Ser fusion – 40 nm from the 5-hydroxytryptophan probe, we conclude that the fluorescence intensity of the probe at position 269 (Fig. 1A) is sensitive the head-to-tail interaction responsible for tropomyosin polymerization.

Fig. 1D presents a scheme for the linear polymerization of tropomyosin in which the 5-hydroxytryptophan probe can exist in one of two environments, (i) a “polymerized” environment (filled circles in the figure, maximum fluorescence intensity), where the C-terminal end of the tropomyosin molecule to which the probe is covalently attached interacts with the N-terminal end of another tropomyosin molecule, and (ii) a free environment (open circles, minimum fluorescence intensity), which occurs only at the C-terminal ends of each tropomyosin polymer and in free monomers. Therefore, the fraction of probes in the free environment is directly proportional to the total polymer concentration (where monomers are considered polymers of chain length (i) = 1). The fraction of probes in each environment is a function of the total tropomyosin concentration and the equilibrium association constant (K) of the head-to-tail interaction under the conditions specified. Oosawa and Kasai (45) show that if we assume that this equilibrium constant is independent of chain length, we can calculate the concentration of each species as a function of the K and the total Tm concentration (see “Experimental Procedures”)

We therefore studied the thermodynamics of the tropomyosin head-to-tail interaction by monitoring the ionic strength dependence of the fluorescence intensity at a number of Tm concentrations, as described under “Experimental Procedures.” Fig. 3A shows representative curves for the ionic strength-dependent changes in fluorescence intensity of AS269(5OHW). At low ionic strength and high Tm concentrations, the normalized (for concentration) fluorescence intensity of the probe is maximum whereas at high ionic strength and low protein concentrations the fluorescence of the probe is significantly reduced. This is also evident in Fig. 3B, where the extent of polymerization (1 – f0) at selected ionic strengths was plotted against tropomyosin concentration. As expected (45), the extent of po-
Ionic Strength Dependence of Tm Head-to-tail Interaction

Fig. 4. A, variation of $K$ for the head-to-tail interaction as a function of ionic strength. In the range for which accurate values for $K$ could be obtained, the data fit the linear relationship $\log K = 11.1 - 18.0[I]^{0.5}$. B, the data are presented as $\log K$ versus $I^{0.5}$. In the range for which accurate values for $K$ could be obtained, the data fit the linear relationship $\log K = 11.1 - 18.0[I]^{0.5}$.

lymerization at a specific ionic strength can be seen to be dependent on the total protein concentration. The data was fit to Equation 8 to obtain the affinity constants for the head-to-tail interaction at a number of ionic strengths (Fig. 4).

In Fig. 4B we see that between [NaCl] = 20 mM ($I = 0.045$) and [NaCl] = 70 mM ($I = 0.095$), the equilibrium constant for the head-to-tail interaction varies by almost 2 orders of magnitude ($1.9 \times 10^3$ m$^{-1}$ and $3.5 \times 10^5$ m$^{-1}$, respectively). At ionic strengths below or above this range, the observed change in intrinsic fluorescence as a function of tropomyosin concentration was too little to accurately estimate $K$. However, a plot of $\log K$ versus $[I]^{0.5}$ reveals an essentially linear relationship (Fig. 4B) from which $K$ at any ionic strength may be interpolated ($\log K = 11.1 - 18.0[I]^{0.5}$). The result can be combined with Equation 7 and $f_\alpha = <i>^{-1}$ to explicitly describe the interdependence of $<i>$, $K$, and total Tm concentration ($\lambda_{\text{tot}}$). This relationship is presented as a three-dimensional surface in Fig. 4A. In Fig. 5B, we see that within the range 0.05 < $I$ < 0.1 and $1 \mu M < [\text{Tm}] < 10 \mu M$, $<i>$ may vary from 1 to 10. Because the relationship between log $K$ and $[I]^{0.5}$ is certainly not truly linear over a wide range of ionic strengths (see Discussion and Refs. 49–51), the $<i>$ values shown in Fig. 5A with $I < 0.05$ should be considered only as rough estimates.

**DISCUSSION**

In this work we have developed a new approach for quantitatively studying Tm head-to-tail polymerization. We have identified a skeletal $\alpha$-tropomyosin mutant with a 5-hydroxytryptophan at position 269 whose fluorescence is sensitive whether or not the C terminus of the protein is involved in a head-to-tail interaction with another Tm molecule. We arrived at this conclusion from a number of independent lines of evidence as follows. (i) The probe fluorescence (normalized with respect to concentration) is sensitive to total Tm concentration in a manner as would be expected for a linear polymerization phenomenon. (ii) The probe fluorescence is sensitive to ionic strength in a manner that parallels the ionic strength dependence of Tm viscosity. (iii) The sensitivity of the probe, which is localized at the C-terminal end of Tm, is dependent on the presence of an Ala-Ser dipeptide fusion at the N-terminal end of the molecule, ~40 nm distant. This dipeptide fusion is necessary for the polymerization of recombinant skeletal Tm expressed in *Escherichia coli* (Fig. 2A and Ref. 19).

The 5-hydroxytryptophan at position 269 is located 15 residues away from the C-terminal end of the molecule (a distance of ~2.3 nm). Several lines of evidence indicate that the head-to-tail interaction involves approximately nine N-terminal residues and nine C-terminal residues from each tropomyosin molecule (29, 52). Because we are employing a tropomyosin with two extra N-terminal amino acids, we may expect the overlap in ASTm and ASTm269(5OHW) to involve the first and last 11 residues of the polypeptide chains. Therefore, in the polymerized state, the probe at position 269 is only one $\alpha$-helical turn away from the C-terminal end of the tropomyosin molecule. There are several possible mechanisms by which this proximity may directly change the microenvironment of the probe and thereby change its fluorescence signal, including direct electromagnetic field effects, the making and/or breaking of hydrogen bonds involving the probe, conformational changes in the local coiled-coil structure, and changes in conformational flexibility. It is interesting that the fluorescence of 5-hydroxytryptophan probes located just a few amino acids further up the Tm primary sequence (positions 267, 263, and 261) are insensitive to Tm polymerization (33). This suggests that any structural changes in the coiled-coil structure induced by tropomyosin polymerization may be limited to the C- and N-terminal ends of the molecule. Of course, because only a small number of probes have been tested, the behavior of probes at other positions in the molecule may force us to revise this view in the future.

The sensitivity of the probe to the polymerization state of the particular C-terminal end to which it is attached has allowed us to quantitatively determine the fraction of Tm molecules involved in head-to-tail reactions under conditions in which the total Tm concentration and ionic strength are varied. As described under "Experimental Procedures," this data can be analyzed according to linear polymerization theory (45, 46) to calculate the intrinsic head-to-tail association constants as a function of ionic strength as well as the average polymer length and polymer length distributions. A valid question regarding our data is to what extent the modifications introduced into the tropomyosin coiled-coil structure (N-terminal dipeptide fusion and 5-hydroxytryptophan at position 269) affect the thermodynamics of the head-to-tail interaction when compared with the wild-type protein. We believe these effects to be relatively small since the 5-hydroxytryptophan does not affect the behavior of ASTm in viscosity assays (Fig. 2), and ASTm is essentially indistinguishable from chicken muscle tropomyosin in viscosity, actin binding, and actomyosin Mg$^{2+}$-ATPase assays (19).

There are only a few reports in the literature to which we may compare our results. Asai (30), using concentration-dependent electric birefringence of rabbit Tm, obtained a value for $K$ of $0.5 - 1 \times 10^8$ m$^{-1}$ at ionic strengths less than 0.02. Linear extrapolation of our results in Fig. 4 to these conditions predicts an association constant greater than $10^8$ m$^{-1}$, a value consistent with the observation that 8 $\mu M$ tropomyosin is significantly polymerized under these conditions (see points at 0 and 20 mM NaCl, 10 mM imidazole, pH 7, in Fig. 2A). Ooi et al. (31) use light scattering of Tm solutions to calculate a value of $\sim 1.2 \times 10^5$ m$^{-1}$ at $I = 0.1$, which is in good agreement with our
Fig. 5. A and B, dependence of the average polymer length \(<i>\) as a function of ionic strength and total Tm concentration. The surface is described by the combination of Equation 7, \(f_\alpha = <i>^{-1}\), and the linear relationship between log \(K\) and \(F^{0.5}\) derived in Fig. 4. The color codes in the legends refer to differences in the value of \(<i>\). Purple: \(<i> = 1\) in part A. The values of \(<i>\) are most accurate for ionic strengths between 0.05 and 0.1, shown in B (see “Discussion”).

Calculated value of 2.5 \(\times 10^7\) M\(^{-1}\) at this ionic strength. Ooi et al. (31) also determine a \(K\) of 2 \(\times 10^4\) M\(^{-1}\) at 1 M salt. Although we did not perform experiments at this ionic strength, inspection of the ionic strength dependence of \(K\) in Fig. 4 suggests a \(K\) of less than unity at \(I = 1.0\). Sano et al. (32) studied the polymerization properties of tropomyosins with Ser or Glu at position 283. Even though these full-length Tms have significantly different viscosities at low ionic strength, the association constants determined by analytical sedimentation equilibrium analysis of their recombinant N- and C-terminal fragments were essentially the same at \(I = 0.043\) (20 mM potassium phosphate, pH 7), 2–4 \(\times 10^4\) M\(^{-1}\). This value is significantly lower than ours (\(K = 2.5 \times 10^5\) M\(^{-1}\) at \(I = 0.043\), Fig. 4). Therefore although all previous attempts to calculate \(K\) of the head-to-tail interaction gave very similar values (2 \(\times 10^5\)–1.25 \(\times 10^6\) M\(^{-1}\)) over a wide range of ionic strengths (0.01–1.0), our results indicate an extremely high sensitivity to ionic strength; for example, between \(I = 0.045\) and 0.095 we observe a drop in the value of \(K\) by almost 2 orders of magnitude (Fig. 4).

This is the first report in which the association constant for the tropomyosin head-to-tail interaction has been explicitly calculated at a large number of ionic strengths. Simple Debye-Hückel theory predicts a strong ionic strength dependence for interactions dominated by electrostatic forces and is consistent with the linear relationship between log \(K\) and \(F^{0.5}\) over a small ionic strength range observed in Fig. 4B. This relationship (log \(K = 11.1 – 18.0/F^{0.5}\)) allows us to estimate \(K\) at any ionic strength within that range. Therefore, this result can be combined with Equation 7 and \(f_\alpha = <i>^{-1}\) to explicitly describe the interdependence of \(<i>\), \(K\), and total Tm concentration (\(\lambda_{tot}\)). This relationship is presented as a three-dimensional surface in Fig. 5. This figure clearly shows how ionic strength and Tm concentration combine to influence average polymer length. Extensive extrapolation beyond the conditions observed in Fig. 4 should be employed with caution since ionic strength-induced conformational changes in tropomyosin may result in significant deviations from the curve. Furthermore, over a large range of ionic strengths, the relationship between log \(K\) and \(F^{0.5}\) is surely not linear (49–51).

The relationship between the thermodynamics of protein binding and ionic strength can be interpreted in terms of the extended Debye-Hückel law to gain information about the effective charge at the interaction interface (51). Here log \(K\) = log \(K_i + 2A_zz_eL^{1/2}(1 + rF^{0.5})\), where \(K_i\) is the association constant at \(I = 0\), \(z_N\) and \(z_C\) are the net effective charges of the N- and C-terminal interfaces involved in the interaction, \(r\) is the effective distance between charged interfaces, and \(A\) and \(B\) are thermodynamic constants (\(A = 0.509\), \(B = 3.291\) nm\(^{-1}\)M\(^{-0.5}\) at 298 K) (51, 53). The data from Fig. 4 were fit to this equation to obtain values for \(K_i\) and the product \(z_Nz_e\) over a range of values for \(r\) between 0 and 10 Å. If \(r\) is assumed to be zero, then the absolute value of the product of the effective charge at the interface is 18 e.s.u.\(^{-2}\) and \(z_N\) is 11.1. These values increase to \(\sim 36\) e.s.u.\(^{-2}\) and 13, respectively, when \(r\) is assumed to be a more realistic value of 5 Å and 61 e.s.u.\(^{-2}\) and 15 when \(r\) is assumed to be 10 Å.

The first and last 15 residues of ASTm are NH\(_3\) - ASMDAIKKKKQMLKL ... and ... ISKELDHALNDMTSI-COO\(^-\), respectively. The tropomyosin head-to-tail overlap is expected to involve approximately nine residues from both the N and C termini of the tropomyosin polypeptide chains (29), but intermolecular salt bridges could presumably involve residues up to one helical turn before and after the overlap region. As mentioned above, in the case of ASTm, we may expect the Ala-Ser N-terminal fusion to extend this overlap to 11 residues (underlined above). A dimer of the first 11 N-terminal residues has 8 positive and 2 negative charges (including the C terminus). Furthermore, lysine and glutamate residues may be found immediately after and before the actual overlap region. Therefore, the N and C termini have several potential charges available to form salt bridges between a highly positively charged N-terminal segment and a highly negatively charged C-terminal segment. The number of charges is more than enough to result in \(z_Nz_e\) values of at least 18 e.s.u.\(^{-2}\), which we observed.

In fact, McLachlan and Stewart (29) present a model for the tropomyosin head-to-tail overlap involving nine residues from each polypeptide terminus that interact via the broad faces of the supercoiled structure. In their model, they proposed three specific intermolecular salt bridges involving the following pairs of residues: His-276–Asp-2, Asp-280–Lys-6, and Ile-
284(COO−)–Lys-7. This would give a maximum $z_2q_2 \cdot e$ value of 9 e.s.u.$^2$, less than our minimum value of 18 e.s.u.$^2$. By manually docking molecular models of the tropomyosin N- and C-terminal coiled-coil structures, built using the recently solved tropomyosin structures as templates (23, 24), we found that in addition to the salt bridges proposed by McLachlan and Stewart (29), the positively charged Lys-12 side chain can point back toward the N terminus of its polypeptide chain to make a salt bridge with the second negatively charged C-terminal carboxylate of the adjacent molecule. In addition, our model also predicts that the side chains of residues His-276 and Asp-280 from one polypeptide chain and Asp-2, Lys-5, and Lys-6 from another may form a network of interdependent intermolecular and intramolecular ionic interactions that would exist only in the context of the head-to-tail complex. None of these newly identified possible ionic interactions (all with interionic distances less than 5 Å) depend on the presence of the N-terminal dipeptide fusion. They would increase the total number of interacting ions to four negatively charged ions and five positively charged ions and increase $z_2q_2 \cdot e$ to 20 e.s.u.$^2$. Ionic interactions over a range of up to 10 Å would further increase the number of interacting ions responsible for the ionic strength sensitivity of Tm polymerization. Clearly, a definitive understanding of this sensitivity awaits the determination of the structure of the head-to-tail complex by high resolution NMR spectroscopy or x-ray crystallography.

Tm binds cooperatively to actin (for review, see Ref. 11). Non-polymerizable tropomyosins produced by peptidase treatment (54), chemical modification (27), or recombinant techniques (18, 19) usually have significantly reduced affinity for actin. This at first suggests that the two phenomena are linked; that is, polymerization promotes actin binding. This idea is consistent with the observation that Tn binding increases Tm viscosity (18, 56) and restores the actin binding ability of non-polymerizable Tm (18, 19, 54, 57). Based on Tn- and S1-induced Tm-actin binding, several reports have concluded that the head-to-tail interaction between Tm molecules contributes more toward intrinsic actin binding rather than the cooperativity of actin binding (58–63). For example, Hill et al. (59) show that Tn enhances Tm binding to actin by increasing the intrinsic affinity of Tm for actin with no increase in cooperativity. If Tm polymerization were the basis of cooperative Tm binding, one would have expected an increase in cooperativity in the presence of troponin. Despite these arguments, due to the low affinity, the cooperativity of non-polymerizable Tm binding to actin in the absence of other proteins has not been measured for comparison with polymerizable tropomyosin. Therefore, whether or not the head-to-tail complex contributes to the cooperativity of actin-binding remains unclear.

Although the head-to-tail complex is apparently of prime importance for actin binding, one would expect that the rest of the tropomyosin molecule also contribute to binding. Measures of this residual affinity are scarce although Moraczewska et al. (55) report an affinity constant of recombinant rat skeletal nTm of $3 \times 10^5 \text{M}^{-1}$ in 100 mM NaCl that drops to much below $10^{-5} \text{M}^{-1}$ at 150 mM NaCl. Our results show that at $I = 0.090$ or 0.190, 2 $\mu$M AS269(50HW) can still bind actin, whereas n269(50HW) does not (Fig. 2B and data not shown). Under these conditions, 40 and 99% of AS269(50HW) molecules, respectively, are expected to be in the monomeric state. The question then arises, if Tm1 is able to bind actin under conditions in which it is essentially in the monomeric state, then why does nTm not bind actin under the same conditions? Because modifications to either the N or C terminus of Tm can modify Tm binding to actin, it seems that it is the actual structure of the head-to-tail complex that confers actin binding and not only the N-terminal portion or C-terminal portion of the molecule. Perhaps the site to which the head-to-tail complex binds on actin further stabilizes it and protects its ionic interactions from destabilization by increases in the ionic strength of the solution. Furthermore, any modification that affects the structure of the N or C terminus would be expected to affect the structure of the complex and, therefore, actin binding affinity as well. The recent high resolution structures of acetylated and unacylated forms of the N-terminal fragments of tropomyosin strongly suggest how $\alpha$-amino acetylation could so strongly affect Tm polymerization and actin binding (22, 23). In the absence of the dipeptide N-terminal extension, repulsions between the positively charged $\alpha$-amino groups at position $\alpha$ in the hydrophobic dimer interface are expected to destabilize the coiled coil structure, which may be important for the stability of the head-to-tail complex responsible for polymerization.

In this work we identified a tropomyosin mutant whose fluorescence reports whether or not the C terminus of the molecule to which it belongs is involved in a head-to-tail interaction with the N terminus of another tropomyosin molecule. We used this property to measure the affinity constant for this interaction at a number of ionic strengths and found that the association constant decreases almost 2 orders of magnitude over a 50 mEq difference in salt concentration. This strong ionic strength dependence indicates the involvement of a large number of charged residues in ionic interactions at the head-to-tail interface. Our analysis allows one to estimate the average polymer length under a specific set of conditions. Because tropomyosin interacts with actin, troponin, and perhaps myosin as well, this parameter may be of importance in the analysis of Tm binding isotherms and help in the interpretation of the thermodynamics and cooperativity of thin filament assembly and the structural transitions induced by the binding of Ca$^{2+}$ and myosin.

**Acknowledgments**—We thank Adriano Paulucci for help with CD measurements, Marcos Alegria for help with viscosity measurements, and Fernando Reinach for helpful discussions.

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J. Biol. Chem. 2002, 277:2081-2088.
doi: 10.1074/jbc.M109568200 originally published online November 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109568200

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