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Folding and stability of a coiled-coil investigated using chemical and physical denaturing agents: Comparative analysis of polymerized and non-polymerized forms of α-tropomyosin

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Abstract

α-Tropomyosin (Tm) is a two-stranded α-helical coiled-coil protein, which participates in the regulation of muscle contraction. Unlike Tm purified from vertebrate muscle, recombinant Tm expressed in *Escherichia coli* is not acetylated at the N-terminal residue and loses the capacity to undergo head-to-tail polymerization, to bind actin and to inhibit actomyosin ATPase activity. These functions are restored by fusion of an N-terminal Ala-Ser (AS) dipeptide tail to recombinant Tm. Here, we have employed chemical (guanidine hydrochloride and urea) and physical (elevated hydrostatic pressures and low temperatures) denaturing agents to compare the structural stabilities of polymeric alanine–serine–tropomyosin (ASTm, containing the AS dipeptide) and dimeric "non-fusion" Tm (nfTm, i.e., not containing the AS dipeptide). Binding of the hydrophobic fluorescent dye bis-ANS, circular dichroism and size-exclusion chromatography were used to monitor the stabilities and state of association of both proteins under different solution conditions. Bis-ANS binding was markedly decreased at low concentrations (<1 M) of GdnHCl or urea, whereas the secondary structures of both ASTm and nfTm were essentially unaffected in the same range of denaturant concentrations. These results suggest local unfolding of bis-ANS binding domains prior to global unfolding of Tm. In contrast, increased bis-ANS binding was observed when Tm was submitted to high pressures or to low temperatures, implying increased exposure of hydrophobic domains in the protein. Taken together, the different sensitivities of ASTm and nfTm to different denaturing agents support the notion that, at close to physiological conditions, head-to-tail interactions in polymerized ASTm are predominantly stabilized by electrostatic interactions between adjacent Tm dimers, whereas non-polar interactions appear to play a major role in the stability of the coiled-coil structure of individual Tm dimers.

Keywords: Tropomyosin; CD; Fluorescence; Guanidine; Urea

1. Introduction

The α-helical coiled-coil is a structural motif found in many fibrous and DNA binding proteins (Cohen & Parry, 1990), and is recognized as a stabilizing struc-

Abbreviations: ASTm, alanine–serine–tropomyosin; CD, circular dichroism; Tm, tropomyosin; nfTm, non-fusion tropomyosin

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ture for pairs of α-helices in proteins. The sequences of coiled-coil proteins are characterized by a heptad repeat of amino acids, usually labeled *a*–*g*, in which positions *a* and *d* are occupied by non-polar residues, whereas the *e* and *g* positions are occupied by acidic and basic residues, respectively (Crick, 1953; McLachlan & Stewart, 1975; Parry, 1975). Both hydrophobic and electrostatic interactions are thought to stabilize coiled-coils (Parry, 1975; Smillie, 1979). Under high ionic strength conditions, when charge screening of electrostatic interactions is expected to occur, hydrophobic interactions have been found to be major determinants of coiled-coil stability (Greenfield & Hitchcock-DeGregori, 1995). However, at physiological ionic strength the relative contribution of electrostatic interactions to the stability of coiled-coils is still not well established (Greenfield & Hitchcock-DeGregori, 1995).

Tropomyosin (Tm) is a two-stranded α-helical coiled-coil protein expressed in nearly all eukariotic cells (Lees-Miller & Helfman, 1991). In striated muscle, Tm is part of the regulatory complex of muscle contraction. α-Tm is a dimer of identical subunits of 284 amino acid residues each. Together with the troponin complex, Tm regulates the interaction between myosin heads and the actin filament (for a review, see Farah & Reinach, 1995). The fact that each Tm molecule makes contact with seven actin subunits along the thin filament suggests that some type of equivalent or quasi-equivalent interaction occurs at each of the seven regions of contact (McLachlan & Stewart, 1976; Parry, 1975; Phillips, Fillers, & Cohen, 1986). Tm dimers are aligned head-to-tail along the length of the actin filament (O'Brien, Bennett, & Hanson, 1971). Structural considerations have led to the proposal that the N- and C-terminal ends of Tm dimers overlap each other by about 8–11 amino acids in head-to-tail interactions (Cohen & Szent-Gyorgyi, 1957; Lehrer, 1975; McLachlan & Stewart, 1975; Palm, Greenfield, & Hitchcock-DeGregori, 2003; Phillips et al., 1986) This overlap would reduce the effective length of Tm from 284 to 273–276 amino acid residues, compatible with the experimentally determined length of individual Tm molecules. Modification or removal of the N- or C-termini of Tm enzymatically or by mutagenesis prevents head-to-tail polymerization and interaction with the actin filament, suggesting that the regulation of actomyosin function by Tm is dependent on head-to-tail interactions (Dabrowska, Nowak, & Drabikowki, 1983; Heeley, Watson, Mak, Dubord, & Smillie, 1989; Hitchcock-DeGregori & Heald, 1987; Mak & Smillie, 1981; Moraczewska & Hitchcock-DeGregori, 2000; Pan, Gordon, & Luo, 1989; Pato, Mak, & Smillie, 1981). Although there is considerable information on the regulatory roles of head-to-tail interactions on the functions of Tm, the effects of polymerization on the stability of individual Tm dimers remains to be better understood.

Unlike Tm directly purified from vertebrate muscle, wild-type recombinant Tm expressed in *Escherichia coli* is not acetylated at the N-terminal residue, which results in loss of the capacity to undergo head-to-tail polymerization, to bind actin and to inhibit actomyosin Mg^{2+}-ATPase activity (Cho & Hitchcock-DeGregori, 1991; Heald & Hitchcock-DeGregori, 1988; Hitchcock-DeGregori & Heald, 1987; Willadsen, Butters, Hill, & Tobacman, 1992). Those functions of recombinant Tm are restored by fusion of an N-terminal Ala-Ser (AS) dipeptide tail to Tm (Maytum, Geeves, & Konrad, 2000; Monteiro, Lataro, Ferro, & Reinach, 1994). Here, we have employed chemical (guanidine hydrochloride, urea) and physical (hydrostatic pressure, temperature) denaturing agents to investigate the structural stabilities of polymeric alanine–serine–tropomyosin (ASTm, containing the Ala-Ser fusion dipeptide) and dimeric "non-fusion" Tm (nfTm, i.e., not containing the AS dipeptide). The results support the notion that, at close to physiological ionic strength conditions, head-to-tail interactions between Tm dimers are predominantly stabilized by electrostatic interactions, while, under the same conditions, hydrophobic interactions play a major role in the stability of the coiled-coil structure of individual Tm dimers.

2. Materials and methods

Bis-ANS was from Molecular Probes (Eugene, OR). Stock solutions of bis-ANS were prepared in methanol and their concentrations were determined using the extinction coefficient, $\varepsilon_{360} = 23{,}000\ M^{-1}\ cm^{-1}$. Urea and guanidine hydrochloride were from Sigma Chem. Co. (St. Louis, MO). All other reagents were of analytical grade.

2.1. Protein expression and purification

The plasmids for bacterial expression of chicken striated muscle α-ASTm and α-nfTm were kind gifts from Dr. Chuck S. Farah (University of São Paulo, Brazil). The recombinant proteins were expressed in *E. coli* strain BL21(DE3) pLysS and purified as previously described (Monteiro et al., 1994). Actin and myosin were purified from chicken *pectoralis major* and *minor* muscles as previously described (Pardee & Spudich, 1982; Reinach, Masaki, Shafiq, Obinata, & Fischman, 1982) and were generously provided by Dr. Martha M. Sorenson (Federal University of Rio de Janeiro, Brazil). Protein concentrations were determined by the method of Hartree (1972).

2.2. Fluorescence measurements

Experiments were carried out in buffer containing 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 6 μM bis-ANS. Data were acquired on a Hitachi F-4500 spectrofluorometer at 25 °C. Bis-ANS spectra were acquired with excitation at 365 nm and emission from 400 to 600 nm. In equilibrium unfolding experiments, samples were incubated for 12 h at 25 °C in the presence of the indicated concentrations of GdnHCl or urea prior to fluorescence measurements.

Fluorescence measurements under pressure were carried out on an ISS (Champaign, IL) PC1 spectrofluorometer using a pressure cell similar to that originally described in (Paladini & Weber, 1981) equipped with sapphire optical windows. The temperature of the pressure cell was controlled by means of a jacket connected to a circulating bath and was monitored by a telethermometer. Bis-ANS spectra were obtained after 10 min of equilibration at each pressure. Control experiments showed that longer equilibration times under pressure caused no further changes in bis-ANS emission.

2.3. Circular dichroism

Far-UV circular dichroism (CD) measurements were performed on a Jasco J-715 spectropolarimeter equipped with a thermostated cell holder. A cylindrical cell with a path-length of 0.1 cm was used. In equilibrium unfolding experiments, samples were incubated for 12 h at 25 °C in the presence of the indicated concentrations of GdnHCl or urea before CD measurements.

2.4. Size-exclusion chromatography

An HPLC system (Shimadzu, Kyoto, Japan) was used with a Superdex 200 HR 10/30 column previously equilibrated in 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA and 1 mM DTT, in the absence or in the presence of GdnHCl or urea (as indicated in Section 3). ASTm or nfTm samples (4 μM, expressed as dimers) dissolved in the same buffer were applied onto the column and the elution (flow rate, 0.7 ml/min) was monitored by intrinsic tyrosine fluorescence emission (excitation at 275 nm and emission at 303 nm).

3. Results and discussion

3.1. Binding of bis-ANS to native ASTm and nfTm

Tropomyosin dimers contain no tryptophan residues and six tyrosine residues per monomer. Tyrosine fluorescence in proteins is often weak and is insensitive to changes in solvent exposure of the fluorophore (Lakowicz, 1983), making the intrinsic fluorescence emission of Tyr residues a poor indicator of protein conformational changes. This led us to use bis-ANS as an extrinsic probe to investigate the stability of Tm under different experimental conditions. Bis-ANS has a very low fluorescence quantum yield in aqueous solution, and displays a marked increase in emission when bound to hydrophobic domains close to positively charged residues in proteins (Rosen & Weber, 1969). Bis-ANS does not normally bind to either native or fully unfolded proteins, as the former do not usually expose significant hydrophobic domains to the solvent and the latter do not exhibit sufficiently organized exposed hydrophobic domains to create a binding site for the dye (for recent examples, see Chapeaurouge, Johansson, & Ferreira, 2001, 2002; Martins, Chapeaurouge, & Ferreira, 2002, 2003). Fig. 1 shows bis-ANS fluorescence emission spectra in the presence of ASTm and nfTm. Interestingly, marked increases in bis-ANS fluorescence were observed in the presence of both ASTm and nfTm when compared to the fluorescence emission of the dye in buffer alone. This suggests the existence of surface-exposed

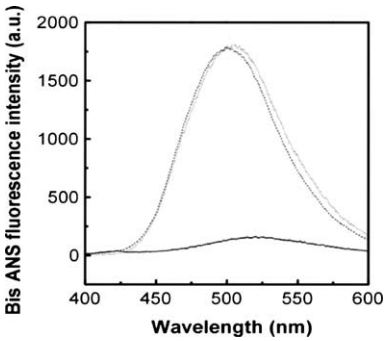


Fig. 1. Bis-ANS fluorescence emission spectra in aqueous buffer (solid line) or in the presence of ASTm (dotted line) or nfTm (dashed line). Proteins (4 μM dimers) were diluted in 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM DTT and 1 mM EDTA. The concentration of bis-ANS was 6 μM. Spectra were measured at room temperature (23 °C). Bis-ANS fluorescence was excited at 365 nm.

hydrophobic domains that act as bis-ANS binding sites in ASTm and nfTm. This is a surprising finding, as native proteins do not usually bind bis-ANS significantly (e.g., Chapeaurouge et al., 2001, 2002; Lopes, Chapeaurouge, Manderson, Johansson, & Ferreira, 2004). It should be noted, however, that isolated troponins I and T in solution were also recently shown to bind bis-ANS (Martins et al., 2002). This suggests that the existence of exposed non-polar domains may be a common property of the proteins involved in the regulation of muscle contraction, and may be implicated in molecular recognition and in the correct assembly of the regulatory complex. Control CD measurements indicated that the secondary structures of ASTm and nfTm (4 μM, expressed as dimers) were unaffected in the presence of up to 6 μM bis-ANS (the concentration used throughout this study; data not shown).

According to an early structural model (McLachlan & Stewart, 1975), head-to-tail interactions between adjacent dimers in the Tm filament are stabilized by three salt bridges and by a number of non-polar interactions between the N- and C-terminal domains of each dimer. Thus, if the non-polar residues present at the N- and C-termini of the molecule were to become exposed to the solvent in the non-polymerized, free Tm dimers one might expect to find increased bis-ANS binding to nfTm relative to ASTm. Interestingly, however, binding of bis-ANS to both polymerized and non-polymerized Tm was virtually identical (Fig. 1). Together with structural data indicating that the first two residues at the N-terminus of Tm are not in a helical conformation (Brown et al., 2001), these results suggest that the N- and C-terminal domains of nfTm are organized in such a way as to bury the non-polar residues at the interface of the coiled-coil.

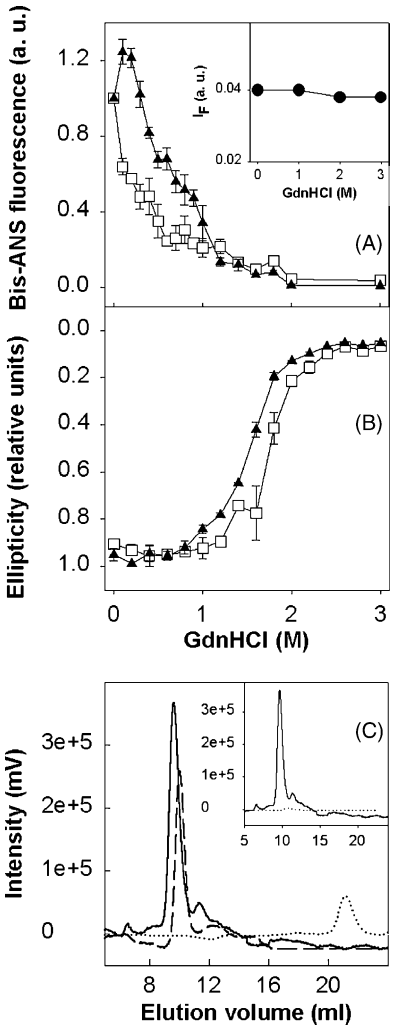
3.2. Effects of GdnHCl on ASTm and nfTm

In order to investigate the interactions that are important for the stability of Tm, we have compared the effects of different chemical and physical denaturing agents on ASTm and nfTm. Comparison between these two proteins allowed discrimination between effects related to inter-molecular interactions (i.e., involving head-to-tail interactions in the ASTm polymer) and intra-molecular effects on the stability of nfTm dimers.

Bis-ANS binding to ASTm was maximal in the absence of denaturant and decreased sharply with increasing guanidine concentrations (Fig. 2A, empty squares). Low concentrations of GdnHCl (≤1 M) promoted a marked (∼80%) decrease in bis-ANS fluorescence, indicating disruption of surface-exposed hydrophobic domains on ASTm and release of bound bis-ANS. Fig. 2A also shows the effect of GdnHCl on bis-ANS binding to nfTm (filled triangles). Interestingly, very low concentrations of GdnHCl (up to 0.2 M) caused a 25% increase in bis-ANS fluorescence, suggesting additional exposure of hydrophobic domains in nfTm. This initial increase was followed by a fluorescence decrease at higher GdnHCl concentrations. The difference in unfolding behavior of ASTm and nfTm may be explained by increased exposure of hydrophobic residues at the N- and/or C-terminal domains of nfTm induced by low (0.1–0.2 M) GdnHCl concentrations. Moreover, addition of 0.3 M GdnHCl to ASTm promoted ∼50% decrease in bis-ANS fluorescence, whereas addition of 0.8 M GdnHCl was required to produce a similar decrease with nfTm. Control measurements showed that increasing concentrations of guanidine hydrochloride had no effect on the very low fluorescence emission of bis-ANS in aqueous buffer alone (Fig. 2A, inset).

As noted above, ASTm and nfTm differ by the fact that ∼8–11 amino acid residues at the C- and N-termini of ASTm are involved in head-to-tail polymerization. This suggests that the hydrophobic residues of nfTm that become exposed and bind additional bis-ANS in the presence of low concentrations of

GdnHCl are those, which are involved in the head-to-tail polymerization of ASTm. It is possible that, in addition to the lack of complete helicity, the internalization of non-polar residues at the N and C-termini of nfTm explains its weak binding to actin and its well-known lack of capacity to regulate actomyosin activity (Brown et al., 2001; Cho, Liu, & Hitchcock-DeGregori, 1990; Dabrowska et al., 1983; Mak & Smillie, 1981; Moraczewska, Nicholson-Flynn, & Hichcock-DeGregori, 1999; Novy, Liu, Lin, Helfman, & Lin, 1993; Pittenger, Kistler, & Helfman, 1995; Sano, Maeda, Oda, & Maeda, 2000; Urbancikova & Hitchcock-DeGregori, 1994).



Changes in the secondary structures of ASTm and nfTm as a function of GdnHCl concentrations were investigated by CD (Fig. 2B). For both proteins, little or no changes in ellipticity at 222 nm were observed up to 1 M GdnHCl. At higher GdnHCl concentrations, ASTm was slightly more resistant than nfTm to unfolding, with full unfolding of both proteins achieved at 2.5 M GdnHCl.

To determine whether GdnHCl caused the dissociation of Tm dimers, nfTm samples were incubated in the absence or in the presence of 1 M GdnHCl and were analyzed by size-exclusion chromatography (SEC) using a column, which was previously equilibrated in the absence or in the presence of 1 M GdnHCl, respectively. The elution volumes of nfTm samples were 9.7 ± 0.1 and 10.0 ± 0.1 ml in the absence or in the presence of 1 M GdnHCl, respectively (Fig. 2C), indicating that the two α-helical strands of nfTm do not undergo dissociation up to 1 M GdnHCl. Control experiments showed that fully unfolded, monomeric Tm (in the presence of 3 M GdnHCl) exhibited an elution volume of approximately 22 ml under the same SEC conditions (Fig. 2C).

It is important to note that, under our experimental conditions, ASTm was fully polymerized. This was indicated by comparative SEC analysis of nfTm and ASTm samples: while nfTm eluted from the column as expected for the native dimeric protein, the ASTm sam-

Fig. 2. Unfolding of ASTm and nfTm by GdnHCl. Experimental conditions were as described in the legend to Fig. 1. After addition of GdnHCl, the samples were incubated for 24 h at room temperature before measurements. ASTm: empty squares; nfTm: filled triangles. *Panel A:* normalized bis-ANS fluorescence (excitation at 365 nm and emission integrated from 400 to 600 nm). The *inset* shows the fluorescence of bis-ANS alone (in the absence of protein) in aqueous buffer as a function of guanidine hydrochloride concentration. The *y*-axis scale in the *inset* is normalized as in the main panel, and reflects the fact that the fluorescence of free bis-ANS in buffer is much lower than when the dye is bound to tropomyosin (see Fig. 1). *Panel B:* normalized ellipticity values from CD measurements at 222 nm. *Panel C:* size-exclusion chromatography of nfTm in the absence (solid line) or in the presence of 1 M GdnHCl (dashed line) monitored by intrinsic fluorescence emission (excitation at 275 nm and emission at 303 nm). The chromatogram of a sample in the presence of 3 M GdnHCl (corresponding to fully dissociated/unfolded tropomyosin) is also shown for comparison (dotted line). *Inset:* SEC analysis of native nfTm (solid line) and ASTm (dotted line) samples (see text). Bis-ANS fluorescence and CD measurements are presented as mean ± S.E.M. of three independent experiments.

ple was completely retained at the top of the gel and did not enter the column (Fig. 2C, inset). Right-angle light scattering measurements were also consistent with a high extent of polymerization of ASTm compared to non-polymerized nfTm (data not shown).

Furthermore, light scattering and SEC analysis showed that exposure to 1 M GdnHCl caused complete depolymerization of ASTm (data not shown).

Interestingly, the release of bound bis-ANS from both nfTm and ASTm observed up to 1 M GdnHCl was not related to overall unfolding of the proteins, as their secondary structures were practically unaffected in this concentration range of denaturant (Fig. 2B). In addition, SEC analysis showed that Tm dimers (nfTm) did not undergo dissociation up to 1 M GdnHCl (Fig. 2C). Thus, the decrease in bis-ANS fluorescence can be attributed to local unfolding of Tm in the neighborhood of bis-ANS binding sites, without secondary structure changes or dissociation of the dimer. Local instability of Tm has also been proposed based on thermal unfolding experiments using pyrene as a fluorescent probe covalently bound to cysteine residues of Tm (Ishii, 1994; Ishii & Lehrer, 1990) or using CD measurements (Greenfield & Hitchcock-DeGregori, 1995).

3.3. Effects of urea on ASTm and nfTm

Fig. 3A shows urea-induced structural changes in ASTm and nfTm monitored by bis-ANS fluorescence. In contrast with the results obtained using GdnHCl as a denaturant, the unfolding transitions of ASTm and nfTm induced by urea were practically identical (Fig. 3). Comparison of the effects of GdnHCl and urea on the local unfolding of Tm (monitored by bis-ANS fluorescence) showed that GdnHCl was significantly more efficient than urea in inducing unfolding of ASTm: the concentration of urea required to promote 50% decrease in bis-ANS fluorescence was 0.8 M, almost three times higher than the concentration of GdnHCl required to produce the same effect on ASTm. On the other hand, for dimeric nfTm the effects of both denaturing agents were quite similar. These results support the notion that electrostatic interactions (which are preferentially destabilized by GdnHCl) play important roles in the stabilization of head-to-tail interactions in the Tm filament, but not as much in the stability of Tm dimers. Control measurements showed that increasing concentrations of urea had no effect on

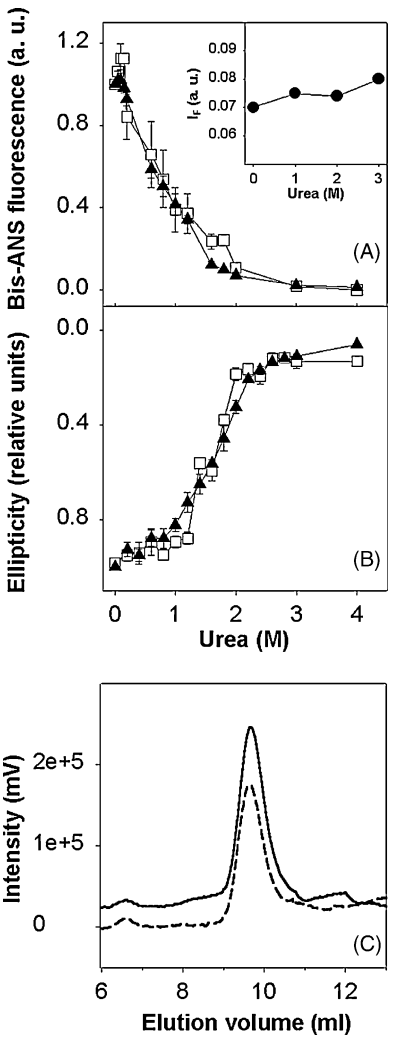


Fig. 3. Unfolding of ASTm and nfTm by urea. Experimental conditions were as described in Fig. 1. Samples were incubated for 24 h at room temperature in the presence of the indicated concentrations of urea before measurements. Empty squares: ASTm; filled triangles: nfTm. *Panel A:* normalized bis-ANS fluorescence intensity (excitation at 365 nm and emission integrated from 400 to 600 nm). The *inset* shows the fluorescence of bis-ANS alone (in the absence of protein) in aqueous buffer as a function of urea concentration. The *y*-axis scale in the *inset* is normalized as in the main panel, and reflects the fact that the fluorescence of free bis-ANS in buffer is much lower than when the dye is bound to tropomyosin (see Fig. 1). *Panel B:* normalized ellipticity values from CD measurements at 222 nm. *Panel C:* size-exclusion chromatography of nfTm in the absence (solid line) or in the presence of 1 M urea (dashed line). Fluorescence and CD measurements are presented as mean ± S.E.M. of three independent experiments.

the fluorescence of bis-ANS in aqueous buffer alone (Fig. 3A, inset).

Despite the significant decrease in bis-ANS fluorescence observed at low concentrations of urea, CD data showed that the secondary structures of both proteins were little affected up to 1 M of this denaturant (Fig. 3B). These observations suggest local unfolding in the vicinity of the bis-ANS binding sites in the presence of low urea concentrations, before any significant changes in the secondary structure of Tm take place. The unfolding transitions of ASTm and nfTm monitored by CD were quite similar, with a mid-point at approximately 1.5 M urea for both proteins. The SEC elution profiles of nfTm in the presence or in the absence of 1 M urea were identical (Fig. 3C), suggesting that the two strands in the coiled-coil were not dissociated up to this concentration of denaturant.

3.4. Stabilities of ASTm and nfTm at low temperatures

Fig. 4A shows bis-ANS fluorescence in the presence of ASTm and nfTm as a function of temperature. In contrast with the results obtained with GdnHCl, ASTm was found to be more stable than nfTm in cold denaturation experiments (Fig. 4). For ASTm, a 60% increase in bis-ANS fluorescence intensity was observed when the sample was cooled from 23 to 1 °C, indicating increased exposure of hydrophobic domains in the protein. Under the same conditions, the increase in bis-ANS fluorescence in the presence of nfTm was much larger (>200%). Control measurements showed that the intrinsic fluorescence emission of bis-ANS alone in aqueous buffer was not affected by decreasing temperature down to 1 °C (data not shown). It is important to note that the secondary structures of both proteins were not affected by decreasing temperature, as indicated by circular dichroism measurements (Fig. 4B). The partial dissociation of Tm dimers and, consequently, the exposure of hydrophobic residues previously involved in non-polar interactions, may explain the increase in bis-ANS fluorescence at low temperatures. This effect suggests a significant contribution of hydrophobic interactions to the stabilization of Tm dimers, in accord with previous results obtained with Tm fragments (Lumb, Carr, & Kim, 1994). Greenfield and Hitchcock-DeGregori (1995) also proposed that the stability of Tm dimers is primarily a function of the hydrophobicity of their residues. However, in the latter study the experiments were carried out in the presence of 500 mM NaCl, which could lead to charge screening and to an underestimation of the electrostatic contribution to dimerization under physiological conditions.

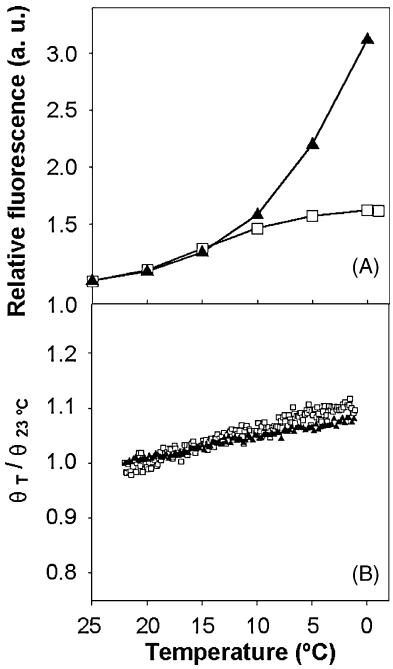


Fig. 4. Stability of ASTm and nfTm at low temperatures. Experimental conditions were as described in Fig. 1. ASTm: empty squares; nfTm: filled triangles. The temperature of the samples was gradually decreased, and measurements of bis-ANS fluorescence intensity (excitation at 365 nm and emission integrated between 400 and 600 nm; *Panel A*) or circular dichroism (ellipticity at 222 nm; *Panel B*) were carried out.

3.5. Pressure-induced depolymerization of ASTm

The effects of hydrostatic pressure on bis-ANS binding to ASTm and nfTm are shown in Fig. 5A (inset). For ASTm, there was a two-fold increase in bis-ANS fluorescence with increasing pressures up to 3.5 kbar, suggesting additional exposure of hydrophobic surface to the solvent. By contrast, no significant effect of pressure on bis-ANS fluorescence was observed in the presence of nfTm. Increasing pressure up to 3.5 kbar had no effect on the fluorescence emission of bis-ANS alone in aqueous buffer (data not shown). One possible explanation for the different behaviors of ASTm and nfTm is that the increase in bis-ANS fluorescence

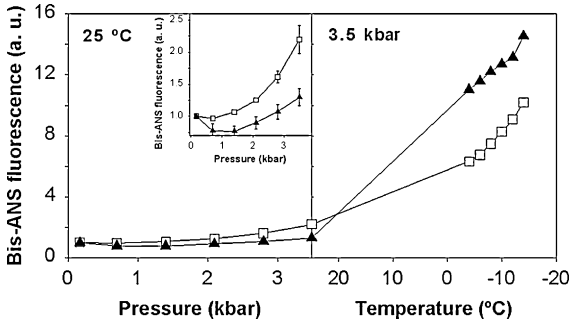


Fig. 5. Pressure and cold denaturation of nfTm and ASTm. ASTm (empty squares) or nfTm (filled triangles) were submitted to increasing pressures at room temperature (23 °C; *Panel A*). After reaching 3.5 kbar, the temperature was gradually decreased to −14 °C (*Panel B*). Samples were monitored by bis-ANS fluorescence intensity measurements (excitation at 365 nm and emission integrated between 400 and 600 nm). Other experimental conditions were as described in the legend to Fig. 1. The results are presented as mean ± S.E.M. of three different experiments. Error bars are smaller than the symbols used.

(with ASTm) is due to pressure-induced depolymerization of tropomyosin filaments and exposure of hydrophobic domains that were previously involved in head-to-tail interactions. The higher bis-ANS binding to pressurized ASTm also may be related to unfolding and/or dissociation of individual dimers. Although our data do not allow distinction between these possibilities, the significant binding of bis-ANS even at 3.5 kbar indicates the presence of exposed organized hydrophobic domains in the structure of Tm, showing that ASTm does not become fully unfolded under those conditions. In this regard, Suarez, Lehrer, and Silva (2001) have proposed the existence of regions of local instability along the Tm molecule under 2.4 kbar of pressure. Those authors proposed that, in such regions, the structure of Tm becomes looser and only locally separated, without full dissociation of the two chains of the dimer.

Pressure is known to shift the equilibrium of a system to the state that occupies a smaller volume, i.e., it favors processes that take place with negative volume changes (Mozhaev, Heremans, Frank, Masson, & Balny, 1996). Electrostatic interactions are, therefore, significantly affected by pressure, since the solvation of free charged groups (electrostriction) is accompanied by a decrease in volume. Consistent with the GdnHCl unfolding data presented above, the greater sensitivity of ASTm to pressure dissociation indicates a significant contribution of electrostatic interactions to the stabilization of head-to-tail interactions. According to McLachlan and Stewart (1975), stabilization of head-to-tail interactions involves three salt bridges (His276-Asp2, Asp280-Lys6 and Ile284 (COO^-)-Lys7) and an extensive network of up to 12 van der Waals contacts between amino acid residues. More recently, Sousa and Farah (2001) suggested that additional salt bridges involving Lys12, His276 and Asp280 from one chain and Asp2, Lys5 and Lys6 from the other chain might participate in a network of ionic interactions in head-to-tail stabilization. Our present data lend support to these previous models by showing that the electrostatic contribution to the stabilization of head-to-tail interactions is indeed significantly higher than the contribution of hydrophobic interactions.

3.6. Pressure-assisted, cold denaturation of ASTm and nfTm

The freezing point of water is significantly depressed at high pressures (Bridgman, 1931), allowing cold denaturation experiments to be carried out at subzero temperatures in the absence of cryoadditives. Pressure-assisted cold denaturation experiments revealed a higher stability of ASTm in comparison to nfTm (Fig. 5). For nfTm, an 11-fold increase in bis-ANS fluorescence (relative to the fluorescence intensity measured at room temperature) was observed when the sample was cooled to −4 °C, whereas with ASTm a further decrease in temperature to −14 °C was required to produce a similar increase in bis-ANS fluorescence. Control experiments showed that, at 3.5 kbar, the fluorescence emission of bis-ANS in aqueous buffer (in the absence of Tm) was unaffected by decreasing temperatures in the range employed in the present study (data not shown).

Disruption of entropy-driver, hydrophobic interactions is a characteristic signature of the process of cold denaturation of proteins (Privalov, 1992). Thus, the higher bis-ANS fluorescence observed upon cold denaturation of nfTm gives further support to the conclusion that Tm dimers are stabilized by hydrophobic interactions between the subunits. It is also important to consider the possibility that, in addition to the exposure of additional binding sites, low temperatures may also affect the affinity of Tm for bis-ANS, resulting in increased or tighter binding of the dye.

In conclusion, by using two forms of Tm differing in their capacities to undergo head-to-tail polymerization, we have separated the contributions of electrostatic and non-polar interactions to the stability of Tm. Our results support the notion that, at physiological ionic strength, formation of the coiled-coil structure of Tm is driven by non-polar interactions. Furthermore, despite the existence of an extensive network of van der Waals contacts between the N- and C-termini of Tm (McLachlan & Stewart, 1975), the results presented here show that head-to-tail interactions between adjacent molecules in polymerized Tm are predominantly stabilized by electrostatic interactions, as predicted by recent models (Sousa & Farah, 2001).

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