The Stability of Tropomyosin, a Two-Stranded Coiled-Coil Protein, Is Primarily a Function of the Hydrophobicity of Residues at the Helix−Helix Interface†

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ABSTRACT: The sequences of coiled coils are characterized by a repeating heptad of amino acids, *abcdefg*, in which the *a* and *d* residues are generally hydrophobic and form the interface between the two α-helices. In this study, rat and chicken α-tropomyosins (α-TMs) have been used as models to determine whether the effects of mutations on the stability of two-stranded coiled coils can be predicted by a simple algorithm. The thermal stabilities of three wild-type muscle α-TMs and nine chimeras, in which the second and/or sixth or ninth coding exons of one α-TM cDNA were replaced with exons from other α-TM cDNAs, with a sequence encoding the GCN4 leucine zipper or a random coil sequence, have been obtained using circular dichroism spectroscopy. Tropomyosin is almost completely helical along its entire length, but there is no correlation of the thermal stability of the α-TMs with the helical propensity of their component amino acids. The stability can be predicted ($P = 0.90$), however, by assigning a weight to every amino acid residue in each sequence, depending on its frequency of occurrence at the *abcdef* or *g* position in a data base of coiled-coil fibrous proteins, and summing all the weights. The correlation improves if only the residues at the *a* and *d* interface are counted ($P = 0.94$). The major factor modulating the thermal stability appears to be the hydrophobicity of the residues at the coiled-coil interface, since there is a high correlation ($P = 0.91$) of the T_M values with the sum of the hydrophobic moments of the residues found at the *a* and *d* positions.

The α-helical coiled coil is a structural motif found in many fibrous and DNA binding proteins (Cohen & Parry, 1990). The sequences of coiled-coil proteins are characterized by a repeating heptad of amino acids usually labeled *a, b, c, d, e, f,* and *g*. The side chains of the residues in the *a* and *d* positions are usually hydrophobic and form the interface between the α-helices of the coiled coil, while the side chains in the other positions are generally hydrophilic and face the solvent. The stability of short coiled-coil peptides is influenced by the helix propensity of the residues comprising the coiled coil (O'Neil & DeGrado, 1990), the hydrophobicity and packing of the residues at the coiled-coil interface (Zhou et al., 1992a−c; Zhu et al., 1993), inter- and intrahelical electrostatic interactions (O'Shea et al., 1993; Zhou et al., 1994), and N-terminal acetylation (Greenfield et al., 1994; Lumb et al., 1994). There have been no studies, however, testing whether the findings from the peptide models can be applied to predict the stability of long coiled-coiled proteins.

The tropomyosins belong to a family of highly conserved α-helical coiled-coil proteins with developmentally regulated and tissue-specific isoforms which differ greatly in stability [e.g., see Woods (1976), Betteridge and Lehrer (1983), Potekhin and Privalov (1982), and Lehrer and Stafford (1991)]. Thus, tropomyosin is an excellent model system in which to study the factors influencing the stability of coiled coils. The source of the diversity of the tropomyosins comes from the existence of multiple genes and from alternative splicing of transcripts of those genes [for a review, see Lees-Miller and Helfman (1991)]. The best studied tropomyosin gene is the one that codes for nine different α-tropomyosin (α-TM)[1] isoforms. The sequences of the 284-residue α-TMs are identical except for the regions encoded by three alternately spliced exons, numbered 2, 6, and 9. For example, smooth and striated muscle α-TMs are identical except for residues 39−80 and 258−284, which are encoded by exons 2 and 9, respectively.

Tropomyosins differ in their actin binding affinity, ability to regulate the actomyosin S1 ATPase, polymerizability, and their stability [cf. Lehrer and Morris (1984), Matsumura and Yamashiro-Matsumura (1985), and Cho and Hitchcock-DeGregori (1991); reviewed by Pittinger et al. (1994)]. One focus of the research in this laboratory has been to determine how changes in the sequences coded by exons 2, 6, and 9 affect tropomyosin function (Cho & Hitchcock-DeGregori, 1991; Hammell et al., 1993, 1994).

In the course of our studies on tropomyosin function, numerous chimeric and mutant recombinant proteins have been made, which vary in stability. The availability of these proteins has provided an opportunity to search for the underlying principles that determine the stability of long coiled-coil proteins. In previous studies, Ishii et al. (1990, 1992) studied several recombinant tropomyosins, including smooth and striated muscle α-TMs and chimeras described

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[1] Abbreviations: CD, circular dichroism; α-TM, α-tropomyosin.

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Table 1: Exon Composition of α-Tropomyosin Variants[a]

residue	exon 2 39−80	exon 6 189−214	exon 9 258−284
rat α-TMs			
2a6b9d (smooth muscle)	a	b	d
2b6b9d (TM2)	b	b	d
2b6b9a (striated muscle)	b	b	a
2a6a9d	a	a[b]	d
2a6a9a	a	a[b]	a
2a6zip9d	a	zip[c]	d
2a6zip9a	a	zip[c]	a
2b6b9da	b	b	da[d]
2b6b9ad	b	b	ad[e]
chicken α-TMs			
C2a6b9a (striated muscle)	a	b	a
C2zip6b9a	zip[f]	b	a
C2rc	rc[g]	b	a

[a] All tropomyosin cDNAs include the same exons 1a, 3, 4, 7, and 8 and encode 284-resisdue TMs. The sequences differences in the alternative exons of the rat α-TMs are shown in Table 2, and the chicken are shown in Table 3. [b] Residues 191−211 are coded by exon 6a, but residues 189−190 and 212−213 are coded by exon 6b. [c] The 25 residues coded by exon 6 are replaced with residues 4−28 from the leucine zipper domain of the GCN4 transcription factor (Table 2). [d] Residues 258−275 are coded by exon 9d and 276−284 are coded by exon 9a. [e] Residues 258−275 are coded by exon 9a and 276−284 are coded by exon 9d. [f] Residues 47−60 of striated muscle α-TM are replaced by residues 2−15 from the leucine zipper domain of the GCN4 transcription factor (Table 3). [g] Residues 47−60 of striated muscle α-TM are replaced by a random coil sequence (Table 3).

by Cho and Hitchcock-DeGregori (1991) as well as native muscle tropomyosins. They found that the α-TMs with sequences coded by both exon 2b and 9a, found in striated muscle α-TM, were more stable than those coded by exon 2a and 9d, found in smooth muscle α-TM, and that the N-terminal and C-terminal halves unfolded relatively independently of each other.

In the present work we have extended the stability studies of Ishii et al. (1990, 1992) to additional α-TMs to determine whether the differences in the stability of the regions coded by exon 2, 6, and 9 can be explained and generalized. The research had the following objectives: first, to ascertain whether changes in the sequence of α-TM mainly affect the enthalpy or entropy of folding; second, to determine if the effects of mutations on stability are local and additive or are cooperative; and finally, to see if the stability can be predicted by a simple algorithm. The results suggest that the primary factor affecting the stability of the coiled-coil tropomyosins is the hydrophobicity of the residues at the *a/d* coiled-coil interface, and that the effects of mutations are mainly local and additive. Interestingly, the differences in stability of the α-TMs do not appear to relate to their functional differences.

MATERIALS AND METHODS

Preparation and Nomenclature of the Tropomyosins. The proteins examined were all recombinant proteins and included rat smooth muscle α-TMs, rat and chicken striated muscle α-TMs, chimeras of rat smooth, striated muscle and nonmuscle α-TMs, and mutant rat and chicken muscle α-TMs, in which all or part of the regions coded by either exon 2 or exon 6 were replaced with either a leucine zipper sequence from the GCN4 transcription factor (Landschultz et al., 1988) or a random coil sequence. The α-TMs used in this work are described in Table 1. The proteins are referred to by the exons which distinguish them from each other. For example, smooth muscle α-TM is named "2a6b9d", and striated muscle α-TM is called "2b6b9a". The designations a, b, etc. refer to the order of the alternatively spliced exons in the α-TM gene [see Lees-Miller and Helfman (1991)]. Most of the proteins were rat α-TMs, but several were chicken, in which case the name is preceded with a "C". The sequence differences in the regions coded by exons 2, 6 and 9 of rat tropomyosin are listed in Table 2, and the sequence differences in the chicken α-TMs are shown in Table 3. The α-TM mutants and chimeras were constructed and purified as described previously (Cho & Hitchcock-DeGregori, 1991; Hitchcock-DeGregori & An, 1994; Hammell & Hitchcock-DeGregori, 1993, 1994). All recombinant α-TMs were expressed in *Escherichia coli* and are unacetylated at the N-terminal methionine.

Circular Dichroism Measurements. Thermal stability measurements were made by following the ellipticity as a function of temperature in 0.5 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, and 0.0005−0.002 M dithiothreitol (Ishii et al., 1990, 1992). Data were collected on an Aviv Model 62D spectropolarimeter equipped with a five sample thermal equilibration chamber. Data at 222 nm were obtained at 0.2 °C intervals with equilibration for 0.4 min at each point at a protein concentration of 0.1 mg/mL. Data were averaged for 10 s at each point. For some samples, melts were also obtained at concentrations 1 mg/mL at 280 nm in 1 cm cuvettes. In these cases, data were obtained at 1 °C intervals from 0 to 60 °C with 1 min equilibration at each point and were averaged for 30 s. To correct for changes in the birefringence of the 1 cm cells as a function of temperature, the ellipticity at 350 nm was subtracted from the ellipticity at 280 nm. In addition, data were collected on each cell containing buffer alone at 280 and 350 nm as a function of temperature, and the unfolding curves were corrected for the contributions of the buffer and cell. For direct comparisons with the unfolding at 280 nm, data were also collected at the same concentration and equilibrating conditions at 222 nm in 1 mm cells.

Determination of the Apparent T_M of Folding and the ΔG of Folding at 20 °C. The folding of many of the tropomyosins exhibited multiple transitions. To compare the overall stability of the proteins, two criteria were used, the apparent T_M of folding at a concentration of 1.5 μM (0.1 mg/mL) and the apparent free energy of folding at 20 °C at concentrations ranging from 1.5 to 15 μM. The apparent T_M is defined here as the temperature at which the ellipticity at 222 nm is at the midpoint between the value found at 0 °C where all of the tropomyosins studied in this work are fully folded, and 60 °C where they all are fully unfolded. The value of this apparent T_M usually does not correspond to the midpoint of the major folding transition.

To evaluate the free energy of folding at 20 °C, it was assumed that the unfolding could be fit by up to three independent helix−coil transitions with dissociation accompanying the helix−coil transition at the highest temperature. The number of resolvable transitions was determined from the number of maxima in the first derivative of the curve of the ellipticity as a function of temperature. The CD data, normalized to a scale of 0 to 1, $\theta_{observed}$, was fit by the following equations:

$$\theta_{observed} = \epsilon_1\alpha_1 + \epsilon_2\alpha_2 + \epsilon_3\alpha_3 \quad (1)$$

Table 2: Sequences of the Regions Encoded by Exons 2, 6, and 9 in Rat α-Tropomyosins[a]

Exon 2 (Residues 39-80)

Residue	40					45					50					55				
Heptad position	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*
2a (smooth)	**L**	**E**	E	D	I	S	A	K	E	**K**	L	**L**	R	A	S	**E**	**D**	**E**	R	**D**	R
2b	**L**	**E**	D	E	L	V	S	L	Q	**K**	K	**L**	K	G	T	**E**	**D**	**E**	L	**D**	K

	60					65					70					75					80
	d	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*
	V	L	**E**	E	**L**	H	K	**A**	E	D	S	**L**	L	A	**A**	D	E	T	**A**	A	K
	Y	S	**E**	A	**L**	K	D	**A**	Q	E	K	**L**	E	L	**A**	E	K	K	**A**	T	D

Exon 6 (Residues 189-213)

Residue	190					195					200					205					210			
Heptad position	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*
6a[b]	**K**	**C**	R	Q	**L**	**E**	**E**	Q	**L**	R	I	M	D	Q	T	**L**	**K**	A	**L**	M	**A**	A	E	**E**	**K**
6b	**K**	**C**	A	**E**	**L**	**E**	**E**	E	**L**	K	T	V	T	**N**	**N**	**L**	**K**	S	**L**	E	**A**	Q	A	**E**	**K**
6zip[c]	**K**	V	E	**E**	**L**	L	S	K	N	Y	H	L	E	**N**	E	V	A	R	**L**	K	K	L	V	G	E

Exon 9 (Residues 258-284)

Residue	260					265					270					275					280				
Heptad position	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*
9a (striated)	D	E	L	Y	A	Q	**K**	L	K	Y	K	A	I	S	E	**E**	**L**	**D**	H	A	**L**	K	D	M	T	S	I
9d	E	K	V	A	H	A	**K**	E	E	N	L	S	M	H	Q	M	**L**	**D**	Q	T	**L**	L	E	L	N	N	M

[a] Identities are shown in boldface. The sequences for the regions coded by exons 2a, 2b, 6a, 6b, 9a, and 9d are from Ruiz-Opazo and Nadal-Ginard (1977) and Wieczorek et al. (1988). [b] Residues 191−211 of are coded by 6a, but residues 189−190 and 212−213 are coded by 6b. [c] The residues coded by exon 6 were replaced with the codons for residues 4−28 from the from the leucine zipper domain of the GCN4 transcription factor (Landschulz et al., 1988).

where

$$\alpha_1 = K_1/(1 + K_1),\ \alpha_2 = K_2/(1 + K_2),\ \text{and}\ \alpha_3 = (4[C]K_3 + 1 - \sqrt{8[C]K_3 + 1})/4[C]K_3 \quad (2)$$

$$K_1 = \exp\{(\Delta H_1/RT)((T/T_{M1}) - 1)\},\ K_2 = \exp\{(\Delta H_2/RT)((T/T_{M2}) - 1)\},\ \text{and}\ K_3 = \exp\{(\Delta H_3/RT)((T/T_{M3}) - 1) - \ln[C]\} \quad (3)$$

ΔH_1 is the enthalpy of folding of the first transition at the lowest temperature, ΔH_2 the enthalpy of folding of the second transition, and ΔH_3 is the enthalpy of folding of the transition at the highest temperature. T_{M1}, T_{M2}, and T_{M3} are the observed midpoints of each transition. ϵ_1, ϵ_2 and ϵ_3 are the extinction coefficients for the circular dichroism change associated with each transition, and [C] is the concentration of two-chain tropomyosin in mole/L. To calculate the values of ΔH and T_M which best described the unfolding, initial values of these parameters were estimated, and the unfolding equations were fit using the commercial program SigmaPlot 5.0 (Jandel Scientific). The ΔS values were determined at the T_M of each transition using the relationships $\Delta G = nRT \ln K$ and $\Delta S = (\Delta H - \Delta G)/T$. These values were then

Table 3: Sequence Changes in Mutants of Recombinant Chicken Striated Muscle α-Tropomyosin

Residue	50							55					60
Heptad Position	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*
C2b6b9a (wild-type)[a]	N	K	**K**	L	K	G	T	E	D	E	V	E	K	Y
C2zip6b9a[b]	E	D	**K**	V	E	E	L	L	S	K	N	Y	H	L
C2rc6b9a[c]	G	D	G	R	E	G	D	G	R	G	E	G	Y	S

[a] The sequence of chicken striated muscle α-TM (C2b6b9a) is from Gooding et al. (1987). Identities of the wild-type and mutant proteins are shown in boldface. [b] Residues 47−60 of α-TM are replaced by residues 2−15 of the leucine zipper domain of the GCN4 transcription factor (Landshultz et al., 1988). [c] Residues 47−60 of α-TM are replaced with a sequence that has a low probability of forming secondary structure.

usedto estimate the ΔG of folding at 20 °C using the relationship

$$\Delta G = \epsilon_1(\Delta H_1 - 293\Delta S_1) + \epsilon_2(\Delta H_2 - 293\Delta S_2) + \epsilon_3(\Delta H_3 - 293\Delta S_3) \quad (4)$$

RESULTS

We have analyzed 12 α-TM variants in order to understand the relationship between amino acid sequence and thermal stability in a coiled-coil protein. All the α-TMs analyzed were 284 amino acids long, and the N-terminal Met is unacetylated since they were produced in *E. coli*. They are all encoded by cDNAs of the α-TM gene but differ in the regions encoded by alternatively spliced exons (Table 1). Exon 2 (exon 2a or 2b) encodes residues 39−80, exon 6 (6a or 6b) encodes residues 189−214, and exon 9 (9a or 9d) encodes residue 258−284. Residues of exon 2 or exon 6 have also been replaced with non-TM sequences to encode the GCN4 leucine zipper (zip) (Landschultz et al., 1988) or a random coil sequence (rc). The TM variants are identified by the exon composition and the origin of the cDNA (chicken or rat). Some of the variants are naturally occurring, and others are synthetic chimeras. The compositions are summarized in Table 1. The sequence differences are given in Tables 2 (rat α-TMs) and 3 (chicken α-TMs).

The Unfolding Properties of α-Tropomyosin Variants

The stability of the α-TMs was measured by examining the ellipticity at 222 nm, a measure of the total helical content (Greenfield & Fasman, 1969), as a function of temperature. These measurements showed that changes in the sequence of α-TM can perturb both the enthalpy and entropy of folding. A local mutation (14 contiguous residues), designed to disrupt the coiled-coil heptad repeat, greatly destabilized a large portion of the protein. However, multiple changes in widely separated domains had additive rather than cooperative effects.

Changes in Sequence Can Alter Both the Enthalpies and Entropies of Folding. The first objective of this work was to determine whether changes in the sequence perturb the enthalpy or entropy of folding of α-TM. Circular dichroism (CD) data were used to estimate the thermodynamic parameters of folding of the 12 α-TMs. As found by others (Woods, 1976; Graceffa & Lehrer, 1980; Potekhin & Privalov, 1982; Ishii et al., 1990, 1992), the CD of the α-TMs examined here exhibited two to three resolvable transitions. The thermodynamic parameters, estimated from the changes in ellipticity at 222 nm as a function of temperature (see Materials and Methods), are summarized in Table 4. The data summarized in Table 4 were all obtained on solutions containing 1.5 μM protein. Measurements of some of the samples were also performed using higher protein concentrations, 7−20 μM (data not shown). Increasing the protein concentration raised the T_M at which the protein dissociated, but the ΔG values were independent of concentration. Using more than the minimum number of transitions needed to fit the data did not appreciably change the estimated free energy of folding at 20 °C. Attempts to model the data with only one unfolding transition, however, resulted in poor fits for all of the α-TMs examined (the square roots of the variances between the experimental and calculated curves were at least doubled), and the estimated enthapies and free energies of folding were both decreased more than 2-fold.

Although the transitions overlap and are difficult to resolve totally using CD, it is clear that some of the sequence changes affect the entropy of folding, while others affect both the entropy and enthalpy of folding. For example, Figure 1A illustrates the equilibrium thermal denaturation of recombi-

Table 4: Estimate of the Thermodynamic Parameters of Folding of Tropomyosin from the Ellipticity at 222 nm as a Function of Temperature[a]

α-TM	ΔH_1	ΔH_2[b]	ΔH_3[b]	T_{M1}	T_{M2}[b]	T_{M3}[b]	ϵ_1	ϵ_2[b]	ϵ_3[b]	ΔG	T_{Mapp}
2a6b9d	−21 (2)		−157 (18)	25 (1)		32 (1)	0.34 (0.04)		0.66 (0.04)	−4.1 (0.1)	32.4 (1.0)
2b6b9a	−19 (1)		−139 (16)	30 (3)		44 (0)	0.42 (0.06)		0.58 (0.07)	−6.3 (0.2)	42.6 (0.5)
2b6b9d	−24	−104	−188	30	39	48	0.39	0.37	0.24	−6.6	40.7
2a6a9d	−19 (3)	−95 (48)	−107 (27)	30 (10)	32 (1)	40 (3)	0.36 (0.10)	0.34 (0.13)	0.30 (0.25)	−3.6 (1.2)	33.8 (1.1)
2a6a9a	−21	−121	−137	28	33	43	0.45	0.19	0.36	−4.8	35.2
2a6zip9d	−18 (3)	−86 (35)	−110 (27)	24 (4)	32 (1)	45 (1)	0.36 (0.09)	0.29 (0.10)	0.35 (0.01)	−3.9 (0.8)	34.5 (1.0)
2a6zip9a	−23 (0)	−71 (0)	−101 (4)	21 (0)	33 (0)	50 (1)	0.19 (0.01)	0.38 (0.01)	0.40 (0.01)	−5.1 (0.1)	36.9 (0.5)
2b6b9da	−25 (0)	−111 (26)	−178 (9)	30 (2.5)	39 (0)	47 (0)	0.46 (0.06)	0.31 (0.09)	0.23 (0.02)	−6.1 (0.5)	38.7 (0.3)
2b6b9ad	−23		−156	31		45	0.41		0.59	−7.6	43.0
C2b6b9a	−19 (1)		−158 (6)	32 (3)		44 (0)	0.36 (0.00)		0.64 (0.01)	−8.0 (0.5)	43.7 (0.1)
C2zip6b9a	−19 (3)	−192 (137)	−196 (72)	23 (1)	36 (8)	45 (1)	0.22 (0.01)	0.31 (0.21)	0.43 (0.20)	−7.4 (0.2)	43.8 (0.4)
C2rc6b9a	−38 (15)	−34 (17)	−191 (7)	19 (1)	25 (7)	41 (0)	0.29 (0.12)	0.26 (0.20)	0.45 (0.19)	−5.8 (0.1)	36.7 (0.5)

[a] ΔH_1, ΔH_2, and ΔH_3 are the enthalpies of folding of the helix−coil transitions in kcal/mol, T_{M1}, T_{M2}, and T_{M3} are the observed midpoint of each transition, ϵ_1, ϵ_2, and ϵ_3 are the fractions of the α-TM molecule folding in each transition, and T_{Mapp} is the temperature at which the ellipticity, normalized to a scale of 0−1.0, is equal to 0.5 at a protein concentration of 0.1 mg/mL (1.5 μM) in 500 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5. ΔG is the free energy of folding at 20 °C. The average deviations of the thermodynamic parameters are given in parentheses when replicate measurements were performed on identical concentrations of protein (1.5 μM), in which case the data are directly comparable. [b] When only two transitions were observed, the values for the second transition are grouped with ΔH_3, T_{M3}, and ϵ_3, which represent the transition, where the tropomyosin coiled coil dissociates into two chains.

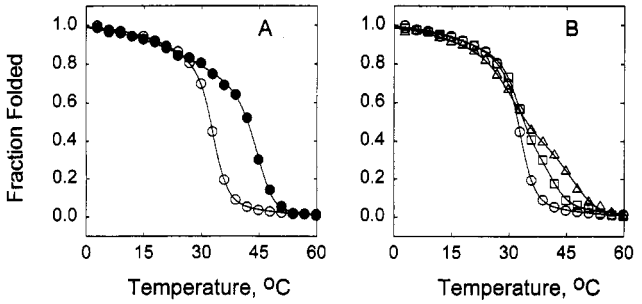

FIGURE 1: Effect of sequence changes in rat α-tropomyosins (α-TMs) on the relative ellipticity at 222 nm as a function of temperature. (A) α-TMs differing in the regions coded by both exons 2 and 9: (○) 2a6b9d, (●) 2b6b9a. (B) α-TMs differing in the region coded by exon 6: (○) 2a6b9d, (□) 2a6a9d, (△) 2a6zip9d. The points are the raw data collected at 0.2 °C intervals (every fifteenth point is illustrated). The lines are the best fits assuming that the data can be described by two to three independent helix−coil transitions, with chain dissociation accompanyng unfolding of the transition with the highest T_M. Conditions: α-TM, 0.1 mg/mL (1.5 μM), in 500 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM dithiotreitol, pH 7.5.

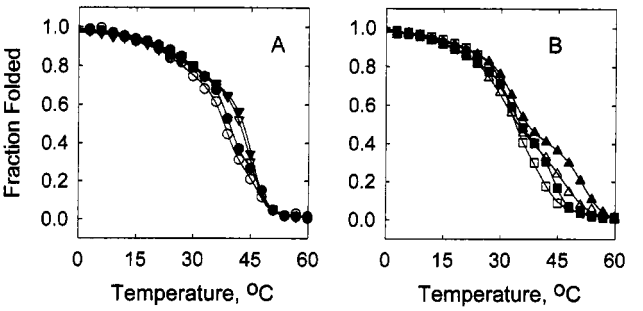

FIGURE 2: Effect of the sequences encoded by exons 6 and 9 on the stability of rat α-tropomyosin. (A) α-TMs differing in the region coded by exon 9: (○) 2b6b9da, (●) 2b6b9d, (▽) 2b6b9a, (▼) 2b6b9ad. (B) α-TMs differing in the region coded by both exon 6 and exon 9: (□) 2a6a9d, (■) 2a6a9a , (△) 2a6zip9d, (▲) 2a6zip9a. Conditions are the same as given in the legend to Figure 1.

nant rat smooth (2a6b9d) and rat striated (2b6b9a) muscle α-TMs, which differ in the regions coded by both exons 2 and 9. The unfolding curves can be resolved into two transitions. As found by others (Ishii et al., 1990), the midpoints of the unfolding curves of smooth and striated α-TM differ by approximately 12 °C. The two proteins exhibit a similar first helix-coil transition, with a T_M of approximately 25−30 °C and a van't Hoff ΔH of approximately −20 kcal/mol, and a second major helix-coil transition with an apparent van't Hoff ΔH value of approximately −130 to −150 kcal/mol (Table 4). The T_M values of the second transition differed, however, suggesting that the differences in stability between the smooth and striated muscle α-tropomyosins are primarily entropic in origin.

When other α-TMs were examined, however, it was clear that changes in the sequence can alter both the enthalpy and entropy of folding. For example, replacing 21 residues (191−211) encoded by exon 6b, in 2a6b9d, with the corresponding amino acids encoded by exon 6a changed only 13 amino acids (Table 2), but greatly perturbed the cooperativity of unfolding (Figure 1B). At least three transitions were needed to fit the data. Curve fitting of the thermal denaturation data suggests that substituting the exon 6 region resulted in a protein which had both lowered entropy and enthalpy of unfolding. The net result is that the protein has a slightly higher apparent T_M of folding (the temperature in which the ellipticity at 222 nm was at the midpoint between the fully folded and fully unfolded states) but a less negative free energy of folding at 20 °C. When a sequence from the coiled-coil leucine zipper, GCN4, was substituted for all 25 residues of exon 6b (6zip), there were 19 nonhomologous substitutions (Table 2). The 6zip replacement increased the T_M but decreased the cooperativity of unfolding relative to 6b, again decreasing both the apparent enthalpy and entropy of unfolding.

The Effects of Changes in the Tropomyosin Sequence Are Additive. Smooth and striated muscle α-TMs differ in the regions coded by both exon 2 and exon 9. While each of these proteins shows one major cooperative unfolding transition, with respective T_Ms of 32 and 43 °C, studies of recombinant fusion and nonfusion tropomyosins and chimeras (Ishii et al., 1990, 1992) have suggested that the domains coded by exon 2 and 9 unfold relatively independently of one another. The work here confirms that the contributions of individual domains to the stability of tropomyosin are independent and also shows that they are additive. For example, Figure 2A illustrates the unfolding of four α-TMs which differ only in exon 9 encoding the C-terminal 27 amino acids. Their cDNAs all included exons 2b and 6b. Tropomyosin 9a is striated muscle tropomyosin and 9d is TM2, a nonmuscle isoform. Two chimeras, 9ad and 9da, contain the first 18 amino acids coded by one exon 9 and the last 9 amino acids coded by the other. The unfolding of both 9a and 9ad exhibits only two transitions; however, the unfolding of 9d and 9da requires at least three transitions to model the unfolding data. The CD data suggest that the region coded by exon 9a has a similar T_M of unfolding as that coded by exon 2b, when examined at a concentration of 0.1 mg/mL, and that they thus both unfold simultaneously in a transition with a T_M of approximately 42 °C. The region coded by exon 9d, however, is less stable than that coded by 9a and unfolds at a lower T_M; thus in 9d an additional unfolding transition is resolved.

It should be emphasized that all of the residues encoded by exon 9 do not contribute equally to the stability of the region. As shown in Figure 2A, the chimera 9ad, in which the last nine residues are encoded by 9d, is actually slightly more stable than 9a, where all of the last 27 residues are encoded by 9a, and 9da is slightly less stable than 9d. The effects on stability of the first 18 residues and the last nine residues coded by exon 9, however, appear to be additive with each other.

When multiple substitutions are made in widely spaced regions of the α-TM sequence, the effects on the stability also appear to be additive. Figure 2B shows the effect of replacing 9d with 9a on the thermal stability of α-TMs, which contain the region coded by exon 2a and either 21 residues of the 6a sequence (2a6a9d) or the GCN4 leucine zipper sequence (2a6zip9d) in the region coded by exon 6 (Table 2). Replacing 9d with 9a increased stability, regardless of the sequence of the exon 6 region, and stabilized half of the molecule approximately 3 °C. The 6zip substitution for 6a increased the T_M and reduced the cooperativity of unfolding, regardless of the identity of exon 9 (Figure 2B).

Disruption of the Heptad Repeat Greatly Disturbs the Cooperativity of Folding. To determine whether disruptions of the heptad repeat have long range effects on the global

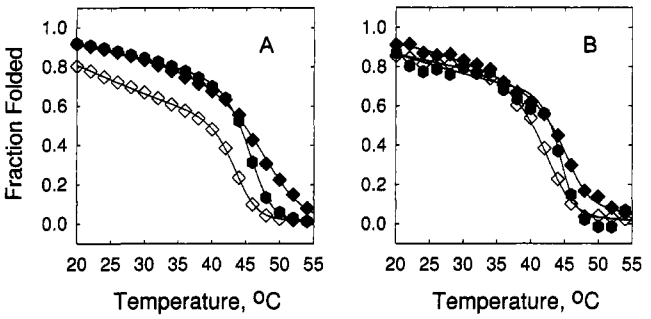

FIGURE 3: Effect of replacing 14 residues coded by exon 2b of chicken striated α-TM (●, C2b6b9a) with a sequence from the GCN4 leucine zipper (◆, C2zip6b9a) or with a random coil (◇, C2rc6b9a) on the relative ellipticity at 222 and 280 nm as a function of temperature. Points were collected at 1 °C intervals. Every second point is illustrated. Conditions: 15 μM α-TM; other conditions are same as given in the legend to Figure 1.

stability of α-TM, two non-TM sequences, one from the GCN4 leucine zipper (2zip), and a random coil sequence (2rc), were substituted for 14 residues (47−60), approximately 5% of the molecule, of chicken striated α-TM in the region coded by exon 2b (see Table 3). The 2zip substitution results in 12 nonhomologous changes, but the heptad repeat is maintained. The 2rc sequence was designed to disrupt the heptad repeat. Disrupting the coiled-coil repeat has a major destabilizing effect on the molecule (Figure 3A); 50% of the molecule unfolds by 37 °C. In contrast, the replacement of the 14 residues with the leucine zipper sequence has only a small effect on the overall stability.

Striated tropomyosin has six tyrosine residues, five of which are in the C-terminal half of the molecule (Gooding et al., 1987). Ishii et al. (1992) have used these tyrosines as probes for the unfolding of the C-terminal half of the molecule. The unfolding of the random coil replacement mutant is monophasic when the ellipticity is followed at 280 nm (Figure 3B), and the mutant appears to be only slightly less stable than 2b (striated). The results suggest that the effect of interrupting the coiled coil by the random coil replacement, while very disruptive, is mainly localized in the N-terminal half of the molecule. The unfolding of the N-terminal and C-terminal domains is, once again, relatively independent.

The thermal denaturation of the α-TMs, like other coiled coils, displays a concentration dependence because they have two chains (Holtzer et al., 1983; Stafford, 1985; Lehrer & Stafford, 1991). The unfolding of 2rc displayed a much more pronounced concentration dependence than either the 2zip or 2b chicken α-TM. The 2rc mutant had both decreased enthalpy and increased entropy of unfolding, the latter suggesting that it is more difficult to bring the two chains together when the heptad repeat is perturbed.

Factors Influencing the Stability of the Tropomyosins

One of the major goals of this study was to determine if the stability of long coiled-coil proteins could be predicted by a simple algorithm. We examined the correlation of several factors with the stability of 12 tropomyosins, including the helical and coiled-coil propensity of the component amino acids, the hydrophobicity and packing of the residues at the coiled-coil interface, and the electrostatic interactions of the side chains.

Changes in Stability Do Not Correlate with the Helix Propensity of the Component Amino Acids. Tropomyosin is helical over almost its entire length (Cohen & Szent-Györgyi, 1957; Phillips et al., 1986). However, the stability of the α-TMs did not correlate with the helical propensity of their amino acid sequences. The helix propensity of the amino acids was assigned using three different scales: (1) the $\Delta\Delta G$ values of stability of each amino acid, derived from the effects of amino acid substitutions in a *f* position on the stability of a model coiled-coil peptide (O'Neil & DeGrado, 1991); (2) the $\Delta\Delta G$ values similarly derived from the effects of host−guest substitutions on the stability of a single-stranded α-helical alanine based peptide (Gans et al., 1991); and (3) the relative probabilities that each amino acid would occur in a helix from a frequency analysis of a protein data base (Chou & Fasman, 1974). The amino acid residues in the sequences of each tropomyosin were assigned weights based on their relative helix propensities and the weights were summed. The percent change in the sum of the weights was calculated relative to the sum of the weights of the amino acids in the sequence of 2a6b9d α-TM, and this change was plotted against the observed T_M of folding, or against the free energy of folding of each chimera at 20 °C (see Materials and Methods). The correlations are summarized in Table 5. The use of all three scales resulted in almost identical estimates of total helix propensity for each tropomyosin. There was very little difference (less than 2%) between the total helical propensity of all the tropomyosins studied, regardless of the scale used, and there was no correlation with stability.

Stability Correlates with the Coiled-Coil Propensity of the Component Amino Acids. Coiled coils are characterized by a heptad repeat with hydrophobic residues at the *a* and *d*

Table 5: Correlation Coefficients of the Stability of α-Tropomyosins as a Function of the Helical Propensity, Coiled-Coil Propensity, and Hydrophobicity of the Residues in Their Amino Acid Sequences

	helical propensity			coiled-coil propensity		hydrophophobicity
method[a]	1[b]	2[c]	3[d]	4[e]	5[f]	6[g]
all residues						
ΔG^h	−0.55	−0.83	−0.35	0.90	0.88	−0.19
$T_M{}^i$	−0.44	−0.76	−0.29	0.91	0.87	−0.10
interface residues						
ΔG	0.65	0.82	0.78	0.94	0.93	0.86
T_M	0.51	0.71	0.68	0.93	0.92	0.91

[a] The helical propensities, coiled-coil propensities, and hydrophobicity of each protein were calculated by assigning a weight to each amino acid in its sequence using the scales described below and summing the weights. The percent change in each sum was calculated relative to that of rat recombinant smooth muscle α-tropomyosin (2a6b9d). The apparent T_Ms and free energies of folding of each protein were plotted as a function of the percent change in weight, and the correlation coefficients of the linear regressions were determined. [b] $\Delta\Delta G$ of helix propensity from host−guest analysis of the unfolding of a model coiled-coil peptide (O'Neil & DeGrado, 1990). [c] $\Delta\Delta G$ of helix propensity from host−guest analysis of the unfolding of an alanine based peptide (Gans et al., 1991). [d] P_α values for the probability an amino acid residue will be helical determined from a data base of 64 proteins (Chou & Fasman, 1974). [e] Average percentage occurence of each amino acid in each position of the heptad repeat of coiled coils (Cohen & Parry, 1990). [f] Relative frequency of each amino acid in heptad repeats (Lupas et al., 1990). [g] Hydrophobic moment of each amino acid (Eisenberg et al., 1982). [h] ΔG is the free energy of folding at 20 °C estimated from the change in ellipticity at 222 nm as a function of temperature. [i] T_M is the temperature at which the ellipticity at 222 nm, normalized to a scale of 0−1, is equal to 0.5.

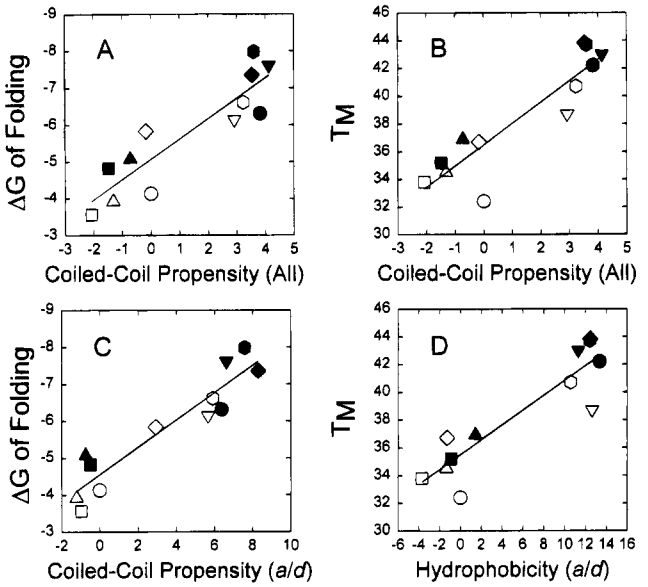

FIGURE 4: Factors affecting the stability of α-tropomyosin. (A) The free energy of folding at 20 °C in kcal/mol and (B) T_M of unfolding of 12 284-residue α-TMs as a function of the percent change in the sum of the coiled-coil propensity of all the residues in their amino acid sequences. (C) The free energy of folding of the α-TMs at 20 °C in kcal/mol, correlated with the percent change in coiled-coil propensity of the *a* and *d* interface residues. (D) The stability of the α-TMs as a function of the percent change of the sum of the hydrophobic moments (hydrophobicity) (Eisenberg et al., 1982) of the *a* and *d* residues. The percent changes in coiled-coil propensity and hydrophobicity are calculated relative to 2a6b9d α-TM. (○) 2a6b9d, (○), 2a6b9a, (●) 2b6b9a, (□) 2a6a9d, (■) 2a6a9a, (△) 2a6zip9d, (▲) 2a6zip9a, (▽)2b6b9da, (▼) 2b6b9ad, (⬢) C2b6b9a, (◇) C2rc6b9a, (◆) C2zip6b9a.

positions. Cohen and Parry (1990) and Lupas et al. (1991) have determined the probability of an amino acid being found in a particular position in the *abcdefg* heptad repeat based on their analyses of data bases of coiled-coil sequences. The sequences of the α-TMs were analyzed to determine if stability correlated with the distribution of their amino acids in the heptad repeat. Each amino acid in the α-TM sequence was assigned a weight based on its frequency of occurrence at each *abcdef* or *g* position, and the weights were summed to determine the "coiled coil propensity" of the 12 proteins. The percentage change in coiled-coil propensity was calculated relative to that of 2a6b9d α-TM. The scales of Cohen and Parry (1990) and Lupas et al. (1991) gave similar coiled coil propensities. There was a high correlation ($P = 0.90$) between the coiled-coil propensity of the 12 α-TMs and both their free energy of folding at 20 °C and their apparent T_M of unfolding (Table 5 and Figure 4A,B). The correlation was slightly better using the percentage occurrence table of Cohen and Parry (1990), which was determined using a data base of two-stranded fibrous coiled coils, than the relative frequency table of Lupas et al. (1991), which examined a greater variety of coiled coils.

Stability Is Primarily Determined by the Hydrophobicity of the Residues at the a/d Interface of the Coiled Coil. When the stability of the α-TMs was correlated with the coiled-coil propensity of the component amino acids, there was a better correlation ($P = 0.94$) when only the amino acids in the *a* and *d* positions were included (Figure 4C). The mean square error between the predicted and found ΔG of folding using the coiled-coil propensity of the interface residues was 0.5 kcal/mol, the same value as the average error of duplicate measurements.

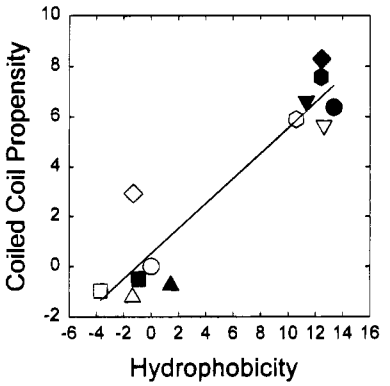

FIGURE 5: Percent change in the coiled-coil propensity of the *a* and *d* residues of 12 α-tropomyosins as a function of percent change in hydrophobicity of the same residues relative to 2a6b9d α-TM (symbols are the same as in Figure 4).

Hodges et al. (1990) showed that the hydrophobic interactions at the coiled-coil interface are the principal factors contributing to stability. The relative hydrophobicities of the *a* and *d* interface residues of the α-TMs were therefore calculated using the "hydrophobic moments" scale of Eisenberg et al. (1982, 1989). There was excellent agreement between the sums of the hydrophobic moments of the *a* and *d* residues and the apparent T_Ms ($P = 0.91$, Figure 4D) and ΔGs ($P = 0.86$) of unfolding. The sum of the hydrophobic moments of the interface residues of the 12 tropomyosins correlated well ($P = 0.92$) with their coiled-coil propensity calculated using the data base of Cohen and Parry (Figure 5). The correlation coefficient improved to 0.97 if the random coil mutant (C2rc6b9a) was not included. The principal factor determining the stability of the tropomyosins therefore appears to be the hydrophobicity of the *a* and *d* interface residues.

DISCUSSION

In this paper we have investigated factors which affect the stability of the α-TMs and have defined a simple algorithm to predict stability based on the amino acid sequence. We found that an excellent predictor for the relative stability of the 12 α-TMs studied is the hydrophobicity of the *a* and *d* residues at the interface between the two α-helices of the coiled coil. The stability of the α-TMs was almost a linear function of the sum of the hydrophobic moments of the interface residues when equilength proteins were compared. Changing only a few of the 82 interface residues has a great effect on the overall stability of the protein. For example, replacing exon 2a in smooth muscle α-TM (2a6b9d) with exon 2b results in a net gain of two hydrophobic residues at the *a/d* interface, K46 and R47 in 2a are replaced by leucines in 2b. There are other changes in the sequence, but they do not affect the net hydrophobicity of the interface. The increased interface hydrophobicity results in an 8 °C increase in T_M and a change in the free energy of folding at 20 °C from −4.1 to −6.6 kcal/mol. In comparing α-TMs with other sequence differences encoded by exon 2, as well as exons 6 and 9, greater stability correlates with a net increase in the hydrophobicity of the *a* and *d* interface residues. The results are consistent with those of Zhou et al. (1992a,b), who found that, in short coiled-

coil model peptides, replacing a single Ala at an *a* or *d* position with a more hydrophobic Leu has an average stabilizing $\Delta\Delta G$ of 3.3 kcal/mol at 20 °C. Therefore, for tropomyosin, hydrophobic interactions are a primary factor influencing protein folding, as in globular proteins [reviewed by DeGrado et al. (1989) and Dill (1990)].

When the coiled-coil propensity of the *a* and *d* interface residues was used to predict stability, as opposed to pure hydrophobicity, there was a slightly better correlation of sequence with the free energy of folding ($P = 0.94$ and 0.86, respectively) (Table 5), suggesting that factors in addition to the hydrophobicity of the interface residues modulate α-TM stability. One possible factor is the packing of the residues at the hydrophobic interface. Harbury et al. (1993) have shown that there is more favorable packing between β-branched amino acids in the *a* positions and leucines in *d* positions than when the positions are reversed, suggesting that packing of the interface residues might affect stability. We were unable to evaluate the role of packing interactions on stability because the four *a/d* pairs with β-branched amino acids at *a* and leucine at *d* positions were invariant in the 12 α-TMs studied. The role of packing in modulating the stability of tropomyosin remains to be tested with specific mutations.

Both inter- and intrachain ionic interactions affect the stability and assembly of short coiled coils and single-stranded α-helices (Marqusee & Baldwin, 1987; O'Shea et al., 1993; Zhou et al., 1994). Zhou et al. (1994) concluded that both intra- and interchain ion pairs contribute approximately 0.4−0.5 kcal/mol each to the stability of a coiled coil at 20 °C. There was no obvious correlation of the number of ion pairs in the α-TMs with stability. The present studies, however, were carried out in 0.5 M NaCl to prevent polymerization of the α-TMs. This high salt concentration would minimize the influence of charge interactions on stability.

Two or three unfolding transitions in the α-TMs were resolved using CD (the number of resolvable transitions was determined by the number of observable maxima in the first derivative of the curve of the change in ellipticity as a function of temperature), whereas calorimetric measurements have shown that tropomyosins may exhibit up to seven independent unfolding domains (Potehkin & Privalov, 1982; Sturtevant et al., 1991). The α-TMs studied had similar pretransitions (25−30 °C), known to correspond to the unfolding of residues 130−190 (Graceffa & Lehrer, 1980; Betteridge & Lehrer, 1983; Ueno et al., 1984), which are identical in all the α-TMs studied. There were one or two major unfolding transitions, depending on the protein. To compare the stabilities of the α-TMs it was necessary to define parameters useful for measuring the overall stability of these multidomain proteins. We used the apparent T_M of folding, which is the temperature at which the ellipticity at 222 nm is at the midpoint between the values of the fully folded and fully unfolded protein, and the ΔG of folding at 20 °C, which was estimated from the change in ellipticity as a function of temperature. The values of the thermodynamic parameters are approximate, because of the difficulty of resolving the thermal transitions. However, even when the transitions were poorly resolved and the estimates of the ΔH_1, ΔH_2, and ΔH_3 values consequently had large errors (e.g., see C2zip6b9a, Table 4), the reproducibility of the calculated ΔG values were very good. The apparent T_M and ΔG proved to be useful for comparative purposes, and it was found the sequences of the α-TMs could be used to predict their values.

The coiled-coil propensity of a given α-TM sequence proved to be a good predictor of stability. The apparent T_Ms were estimated ±1 °C, and the ΔG of folding at 20 °C could be predicted ±0.5 kcal/mol. When extending these studies to other coiled-coil proteins, however, it should be noted that all of the α-TMs that were analyzed in this paper were of equal length. It was difficult to predict the stability of deletion and truncation mutants by comparing their total helical propensity with that of full length tropomyosin (N.J.G., unpublished results) since chain length effects make a large contribution to the stability of coiled-coils (Lau et al., 1984; Qian, 1994, Holtzer et al., 1995) as well as helices in general (Zimm & Bragg, 1959; Sholtz et al., 1991).

In conclusion, the stability of the two-chain α-helical coiled-coil protein, tropomyosin, is primarily a linear function of the hydrophobicity of the residues at the coiled-coil interface, and changes in the interface residues can affect both the enthalpy and entropy of folding. The effects of mutations on the overall stability of α-TM appear to be additive, since the overall stability can be predicted by summing the contributions of each residue at the coiled-coil interface. Changes in the inter- and intrachain ionic interactions of the amino acid side chains appear to make relatively small contributions to the stability of the protein, at least when the α-TMs are examined at high ionic strength.

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