A Scanning Calorimetric Study of Unfolding Equilibria in Homodimeric Chicken Gizzard Tropomyosins

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ABSTRACT Using both circular dichroism (CD) and differential scanning calorimetry (DSC), several laboratories find that the thermal unfolding transitions of $\alpha\alpha$ and $\beta\beta$ homodimeric coiled coils of rabbit tropomyosin are multistate and display an overall unfolding enthalpy of near 300 kcal (mol dimer)$^{-1}$. In contrast, an extant CD study of $\beta\beta$ and $\gamma\gamma$ species of chicken gizzard tropomyosin concludes that their unfolding transitions are simple two-state transitions, with much smaller overall enthalpies (98 kcal mol^{-1} for $\beta\beta$ and 162 kcal mol^{-1} for $\gamma\gamma$). However, these smaller enthalpies have been questioned, because they imply a concentration dependence of the melting temperatures that is far larger than observed by CD. We report here DSC studies of the unfolding of both $\beta\beta$ and $\gamma\gamma$ chicken gizzard homodimers. The results show that these transitions are very similar to those in rabbit tropomyosins in that 1) the overall unfolding enthalpy is near 300 kcal mol^{-1}; 2) the overall ΔC_P values are significantly positive; 3) the various transitions are multistate, requiring at least two and as many as four domains to fit the DSC data. DSC studies are also reported on these homodimeric species of chicken gizzard tropomyosin with a single interchain disulfide cross-link. These results are also generally similar to those for the correspondingly cross-linked rabbit tropomyosins.

INTRODUCTION

Native tropomyosin molecules have an extremely simple structure, the two-stranded coiled coil, wherein two right-handed α-helices are arranged in parallel and register and given a slight left-handed super-twist (Cohen and Parry, 1990). Not only is this same structural motif found in many biological contexts, but its simplicity recommends it as a model for protein folding. The simple structure displays the same hydrogen-bonding, hydrophobic, and electrostatic interactions characteristic of all protein structures, but in a relatively strict, linear geometric context. This coiled-coil conformation results from a pseudo-repeating heptad of amino acids (*abcdefg*) in which residues *a* and *d* are hydrophobic and *e* and *g* bear opposite charge (McLachlan and Stewart, 1975).

In spite of this structural simplicity, there is disagreement concerning the population of macromolecular states coexisting at equilibrium when the coiled coil is unfolded thermally. The most extensively studied tropomyosins are those from rabbit muscle (R-Tm) from which two homodimeric forms have been studied, $\alpha\alpha$- and $\beta\beta$-R-Tm. It is generally accepted that the evidence for multiple states in unfolding equilibria in both these R-Tm species is incontrovertible, but workers differ on how many such states there are and on their precise nature (Lehrer, 1978; Privalov, 1982; Holtzer et al., 1983, 1990; Sturtevant et al., 1991).

Recent CD studies of two homodimeric tropomyosins from chicken gizzard (CG-Tm) have generated further controversy. One study reports that the CD versus temperature data for $\beta\beta$-CG-Tm and $\gamma\gamma$-CG-Tm, each at a single concentration, are well fit by an all-or-none model that supposes the system to consist of only two types of macromolecules: intact dimeric coiled coils and monomeric random coils (Lehrer and Stafford, 1991). However, a second CD study investigates the concentration dependences of the melting temperatures and finds them to be incompatible with the enthalpies of unfolding obtained from the fits in the earlier study (Wrabl et al., 1994). Those reported enthalpy changes are far smaller than corresponding values for R-Tm and lead to a predicted dependence of melting temperature on concentration that is far larger than found.

To settle these questions as to the magnitude of the enthalpy of unfolding of CG-Tm species and the appropriateness of the simple two-state model for CG-Tm, we have performed differential scanning calorimetric (DSC) studies of both $\beta\beta$- and $\gamma\gamma$-CG-Tm. We employ the most common near-neutral saline phosphate buffer medium used in tropomyosin studies: $NaCl_{500}NaPi_{50}EDTA_{10}(7.4)$, wherein we designate complex aqueous media by the formula or abbreviation of each solute, with millimolarity as subscript, followed by pH in parentheses. As will be seen below, the results indicate that these CG-Tm unfolding transitions are rather similar to those in their R-Tm counterparts in that they do not fit a simple two-state model, and the overall enthalpies of unfolding are comparable to those in the R-Tm case. The fact that these enthalpies of unfolding are much larger than those reported by Lehrer and Stafford (1991) suggests that the derivation of thermodynamic data from spectroscopically observed unfolding curves is difficult for complex transitions such as those encountered here.

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Because each homodimeric CG-Tm species can be disulfide-cross-linked at a single site (C36 in $\beta\beta$ and C190 in $\gamma\gamma$ species), and such cross-linking has effects on the unfolding as studied by CD (Wrabl et al., 1994), we have also examined the unfolding of both cross-linked species by DSC and report our findings below. Here again, the results are rather similar to those found earlier for R-Tm (Privalov, 1982; Sturtevant et al., 1991).

MATERIALS AND METHODS

Materials

$\beta\beta$- and $\gamma\gamma$-CG-Tm in both reduced and cross-linked forms were prepared as previously described (Wrabl et al., 1994). For calorimetry, each sample was dissolved in $NaCl_{500}NaPi_{50}EDTA_{10}(7.4)$ and dialyzed exhaustively against this saline buffer solvent at 4°C. The protein solutions were filtered through a 0.45-μm filter before the concentrations were determined spectrophotometrically, using $\epsilon_{\lambda max} = 0.209$ cm^2 mg^{-1} for the $\beta\beta$ form and $\epsilon_{\lambda max} = 0.157$ cm^2 mg^{-1} for the $\gamma\gamma$ form (Wrabl et al., 1994). In addition to the above components, the solvent for the reduced samples contained 5 mM dithiothreitol, added after determination of the protein concentrations.

Calorimetric methods and data analysis

DSC measurements were carried out with the DASM-4 instrument (Biopribor, Puschino, Moscow Region, Russia; see Privalov, 1980) at a scan rate of 1 K min^{-1}. The reference cell was filled with solvent and the instrumental baselines were determined with solvent filling both cells. Protein samples in the concentration range of 1.4–2.0 mg ml^{-1} were studied. Rescanning of the samples indicated essentially complete reversibility provided that the first scan was not carried beyond approximately 95% completion.

All calorimetric data, with instrumental baseline deducted, were analyzed by a nonlinear least-squares curve-fitting procedure outlined elsewhere (Sturtevant, 1987). The model assumed in the curve fitting is that of independent domains, each unfolding in a two-state manner, except that the van't Hoff enthalpy, ΔH_{vH}, which controls the temperature variation of the equilibrium constant for each step, is allowed to vary independently of the calorimetric enthalpy, ΔH_{cal}, for that step, but with the same temperature dependence. Thus there are three independently adjustable parameters for each domain: $t_{1/2}$, the temperature in °C at which the unfolding is half completed; Δh_{cal}, the calorimetric specific enthalpy; and the ratio $\beta = \Delta H_{vH}/\Delta h_{cal}$.

It was found that to obtain adequate fits, i.e., with standard deviation between calculated and observed points amounting to no more than 2% of the maximum value of the excess heat capacity, two domains are enough with the reduced $\beta\beta$ form; but three are required with the cross-linked $\beta\beta$ and reduced $\gamma\gamma$ forms, and four with the cross-linked $\gamma\gamma$ form. For most domain transitions, the parameter β is very near 66,000 g mol^{-1}, the molecular weight of the coiled-coil dimer. However, for both of the reduced homodimeric proteins, a slightly better fit is obtained for the highest melting domain if the parameter β is approximately half the molecular weight of the two-stranded protein. This suggests, but does not prove, that the two α-helical chains separate when the final domain unfolds. For the cross-linked $\gamma\gamma$ form (Fig. 3) a satisfactory fit is obtained with three domains if β for the third domain is taken equal to 33,000, but this is not acceptable for a nondissociating molecule.

With each of the proteins studied there is a significant difference between the heat capacities of the folded and unfolded forms. The temperature variation of enthalpy implied by these heat capacities was included in the curve fitting calculations, the change in the unfolding heat capacity of each domain being assigned a value at the $t_{1/2}$ for that domain in proportion to its enthalpy.

We recognize that the assumption of strictly noninteracting domains is perhaps unrealistic in view of the highly cooperative nature of protein structures. However, inclusion of domain interaction necessitates the introduction of parameters in addition to the three needed, per domain, in the simple model; the precision of the data does not warrant such elaboration.

RESULTS

Figs. 1–4 show typical DSC curves; each figure presents data for one of the two homodimeric GC-Tm species in the reduced or in the disulfide-cross-linked state. Also illustrated are the results of the curve fitting procedure outlined above. In each case, the solid curve represents the observed data, the open circles the calculated points, and the dashed curves the individual domain transitions and the calculated chemical baseline. The latter is determined as the change from the linearly least-squared pre-transition baseline to the linearly least-squared post-transition baseline in proportion to the enthalpy of the transition.

The results of the curve fitting are given in Table 1. The uncertainties listed in the table are the standard errors of the means. If the uncertainty in the determination of the protein concentrations is included, the overall accuracy of the enthalpy data is estimated to be 10%.

Column 2 in the table gives the mean temperature of half-completion, $t_{1/2}$, for each domain; column 3 the mean enthalpy; column 5 the mean value of $t_{1/2}$ weighted by the enthalpy values; column 6 the total enthalpy of unfolding; column 7 the total change in heat capacity at the weighted mean temperature; and column 8, the standard deviation of the calculated points from the observed data expressed as a percentage of the maximum excess heat capacity. Column 4 gives the mean values of β; for the cross-linked samples β was allowed to vary, except that values for the various domains were constrained to be equal. For the reduced

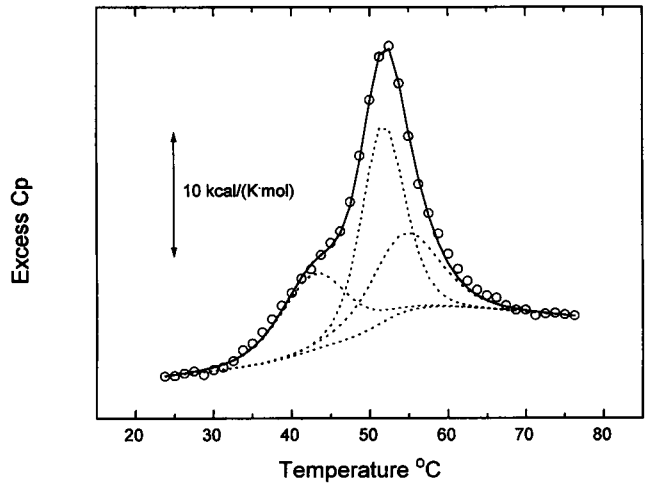

FIGURE 1 DSC curve for cross-linked $\beta\beta$-CG-Tm, showing resolution into three components. Protein concentration 1.62 mg ml^{-1}. Parameters from curve fitting: $t_{1/2} = 42.32, 51.50, 54.00$°C; $\Delta h_{cal} = 1.18, 1.80, 1.10$ cal g^{-1}; $\beta = 66{,}000, 65{,}950, 65{,}950$ g mol^{-1}. Standard deviation of 43 calculated points from observed points = 1.8% of maximum C_p.

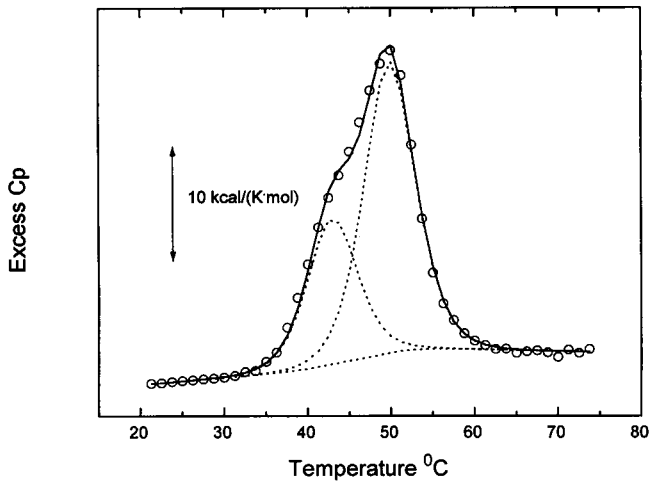

FIGURE 2 DSC curve for reduced $\beta\beta$-CG-Tm at 1.35 mg ml^{-1} showing resolution into only two components. Derived parameters: $t_{1/2}$ = 42.86, 49.80°C; Δh_{cal} = 1.52, 3.10 cal g^{-1}; β = 65,100, 32,800 g mol^{-1}. Standard deviation of 43 calculated points from the observed points = 1.3% of maximum C_P .

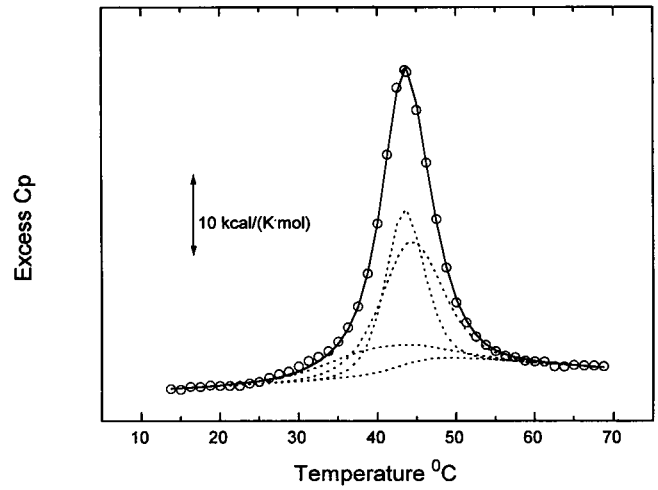

FIGURE 4 DSC curve for reduced $\gamma\gamma$-CG-Tm at 1.8 mg ml^{-1}, showing resolution into three components. Derived parameters: $t_{1/2}$ = 36.50, 43.27, 44.64°C; Δh_{cal} = 0.83, 1.90, 2.38 cal g^{-1}; β = 63,200, 65,800, 32,200 g mol^{-1}. Standard deviation = 1.1% of maximum C_P.

samples, this restriction was not applied to the highest melting domain.

The usual procedure for evaluating the heat capacity change in the thermal unfolding of a protein is to vary the temperature of the unfolding by varying the pH and then taking ΔC_P as the slope of the (usually linear) plot of ΔH versus $t_{1/2}$. Because the unfolding of tropomyosin is not very pH dependent near pH 7 and we had limited amounts of proteins for our experiments, we can only report the overall values for ΔC_P observed in each experiment as the difference in level of the pre- and post-transition baselines at the weighted mean temperature. Values obtained in this way are generally less accurate than those obtained by varying the pH of transition, largely because of uncertainties in the slopes of the baselines.

It is immediately evident from the data themselves (solid curves of Figs. 1–4) that the unfolding of these tropomyosins involves more than a single two-state equilibrium. Only the reduced form of $\gamma\gamma$-CG-Tm exhibits a DSC curve that is not obviously non-two-state. Detailed analysis shows that, even in this case, three transitions are required for the best fit of the data.

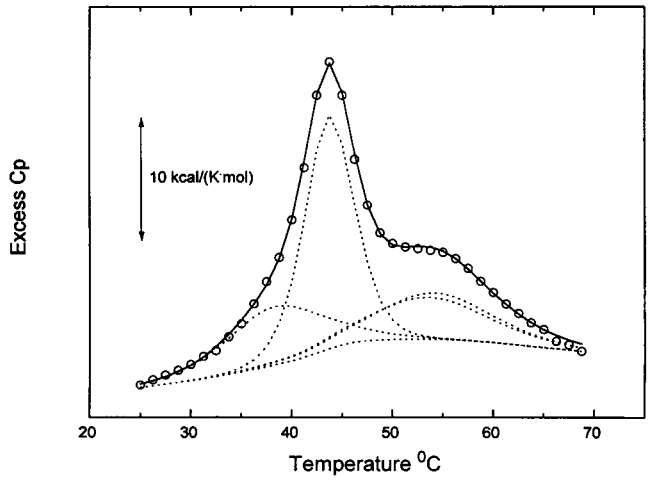

FIGURE 3 DSC curve for cross-linked $\gamma\gamma$-CG-Tm at 1.90 mg ml^{-1}, showing resolution into four components. Derived parameters: $t_{1/2}$ = 37.86, 43.64, 53.39, 54.00°C; Δh_{cal} = 0.90, 1.84, 0.80, 0.85 cal g^{-1}; β = 66,050, 66,100, 66,100, 66,100 g mol^{-1}. Standard deviation = 0.9% of maximum C_P. Here, three components give as good a fit, but only if β = 30,000 for the last component, an impossibility for two cross-linked chains.

DISCUSSION

Because native tropomyosin is in the reduced state, we consider those species first. The two homodimeric variants of R-Tm, $\alpha\alpha$ and $\beta\beta$, are composed of chains that differ at 39 sites, mostly by conservative substitutions. In reduced form, these two variants unfold thermally in a very similar manner (Holtzer et al., 1983; Isom et al., 1984; Sturtevant et al., 1991). Their melting temperatures are similar, and neither fits the all-or-none unfolding model. As shown by DSC (Privalov, 1982; Sturtevant et al., 1991), both have enthalpies of unfolding near 300 kcal mol^{-1} (mol meaning mol of dimer); and both display significant and similar heat capacity changes (1.5–2.6 kcal K^{-1} mol^{-1}).

It therefore is somewhat surprising that a careful CD study of the two corresponding homodimeric forms of CG-Tm, $\gamma\gamma$ and $\beta\beta$, leads to substantially different results between those species, on the one hand, and between them and their R-Tm counterparts on the other. Unlike R-Tm species, both homodimeric forms of CG-Tm yield CD thermal unfolding curves at a single concentration that fit quite well an all-or-none model with zero heat capacity change upon unfolding (Lehrer and Stafford, 1991). Moreover, the resulting enthalpy values, 98 kcal mol^{-1} for $\beta\beta$ and 162 for

TABLE 1 Summary of thermodynamic data for γγ- and ββ-chicken gizzard tropomyosin*

Comp. no.	$t_{1/2}$ (° C)	Δh_{cal} (cal g^{-1})	β (g mol^{-1})	Weighted mean temperature (°C)	$\Sigma\Delta H_{cal}$ (kcal mol^{-1})	$\Sigma\Delta Cp$ (kcal $K^{-1}mol^{-1}$)	SD (% of C_{max})	No. of experiments
Reduced ββ-CG-Tm								
1	42.67 ± 0.32	1.55 ± 0.09	62700 ± 4500	42.66 ± 0.32	299 ± 18	2.82 ± 1.66	1.67	4
2	49.62 ± 0.17	2.98 ± 0.19	33800 ± 2800	49.62 ± 0.17				
Oxidized ββ-CG-Tm								
1	42.63 ± 1.04	0.99 ± 0.11						
2	51.58 ± 0.11	1.75 ± 0.03	65600 ± 150	50.23 ± 0.55	253 ± 9	2.40 ± 1.52	1.92	4
3	54.19 ± 0.49	1.30 ± 0.31						
Reduced γγ-CG-Tm								
1	37.15 ± 1.58	0.76 ± 0.07						
2	43.27 ± 0.08	1.78 ± 0.17	65600 ± 5300	42.80 ± 0.67	320 ± 19	3.74 ± 0.93	1.07	3
3	44.30 ± 0.31	2.31 ± 0.07	33800 ± 1900					
Oxidized γγ-CG-Tm								
1	38.15 ± 0.21	0.97 ± 0.10						
2	43.67 ± 0.06	1.85 ± 0.04	65600 ± 300	46.45 ± 0.22	300 ± 17	2.60 ± 0.90	1.67	4
3	53.45 ± 0.07	0.84 ± 0.08						
4	54.59 ± 0.54	0.90 ± 0.05						

*Listed uncertainties are standard errors of the mean.

γγ, differ drastically from one another, and each is far smaller than that cited above for either R-Tm species.

Such differences, however, are not impossible a priori. The β and γ chains of CG-Tm differ at 72 sites of a total of 284 and thus differ from one another more than do the chains of R-Tm. Moreover, the β chains of R-Tm and CG-Tm differ from one another at 44 sites and the α chain of R-Tm differs from the γ chain of CG-Tm at 60 sites. Although these mutations are mostly conservative, they conceivably could give rise to the behavioral differences described.

However, the all-or-none model with constant heat capacity requires that the enthalpy be directly and simply related to ΔT_m, the change in melting temperature over the full range of protein concentration covered (Lehrer and Stafford, 1991; Wrabl et al., 1994). The small enthalpies required to fit the CD data to the model lead to predictions of rather large values of ΔT_m, 9.2°C and 5.5°C for ββ and γγ, respectively, over the 100-fold range of concentration feasible in CD measurements; yet, a subsequent investigation of the concentration dependence of unfolding for CG-Tm species yields far smaller respective values, 2.9°C and <2°C (Wrabl et al., 1994).

The DSC results reported above clear up the question. Neither reduced homodimeric species of CG-Tm fits an all-or-none unfolding model; the heat capacity change is not zero; and, most important, the overall unfolding enthalpy for each CG-Tm species is near 300 kcal mol^{-1}, about the same as in each homodimeric species of R-Tm. That this larger enthalpy actually does clear up the difficulty concerning the concentration dependence can be seen by a simple calculation, because experience with R-Tm indicates that insertion of the correct overall calorimetric enthalpy into the all-or-none relation provides a fairly good estimate of ΔT_m in spite of the model's deficiencies (Wrabl et al., 1994). So used, the calorimetric enthalpy for CG-Tm species predicts a ΔT_m of 3°C for both, which does not compare badly with the observed values of 2.9°C and <2°C for ββ and γγ, respectively. The small remaining discrepancy in the γγ-CG-Tm case could easily be due to the failure of the all-or-none and/or zero heat capacity assumptions. The ΔC_P values are not only not zero, but are actually slightly larger than those for R-Tm.

The improvement found in the fits to our calorimetry data when the molecular weight is halved in the last unfolding step suggests that the two chains for both homodimeric CG-Tm species separate upon maximum unfolding. Unfortunately, it is not feasible to cover a large enough range of concentration in DSC for a definitive proof. In the case of the ββ species of CG-Tm, the concentration dependence seen in CD, a method that allows a very large range to be covered, leaves little doubt that chain dissociation accompanies unfolding (Wrabl et al., 1994). The case of γγ-CG-Tm is less certain, because concentration dependence is not seen in CD, although experimental limitations allow one to distinguish differences in melting temperature only if they exceed 2°C (Wrabl et al., 1994). Because the entire feasible concentration range of DSC is at the upper limit of the 100-fold range possible in CD for the γγ species, one can conclude only tentatively that dissociation probably also occurs here. Definitive experimental demonstration of dissociation in this case will probably require measurements, by equilibrium ultracentrifugation or by light scattering, of molecular weight as a function of temperature.

Although the overall picture is similar in these homodimeric R-Tm and CG-Tm species, intriguing differences do exist. The simplest transition of all seems to be that for reduced ββ-CG-Tm, which is well fit using only two putative domains. This implies somewhat greater cooperativity in this species of homodimer. The apparent simplicity

of the single-peaked transition for reduced γγ-CG-Tm is deceptive, because it actually requires three domains for the best fit. The similarity in the melting temperatures of these three domains suggests that γγ-CG-Tm is perhaps more uniform in its local stabilities than other tropomyosins, a hypothesis addressable by examining subsequences. Many such studies exist for R-Tm, but none exist for CG-Tm. However, one must also be aware that domain fits employed in DSC studies are not unique. Other multidomain models likely also can fit such data (Skolnick and Holtzer, 1986; Holtzer et al., 1990; Privalov, 1982).

The DSC data for the cross-linked species present a similar picture. Although differences in detail exist, all R-Tm and CG-Tm homodimeric species show several intermediate states and substantial and comparable values of ΔH and ΔC_P. Such differences as exist must stem from differences in local stability and the manner in which they interact with loop entropy effects brought into play because of the presence of the cross-link (Skolnick and Holtzer, 1986). Other differences may stem from the presence in ββ-R-Tm of two cross-links, one at C36 and one at C190, rather than only one (Holtzer et al., 1986).

For all four of the tropomyosin homodimers (counting both rabbit and chicken proteins), interchain disulfides link residues C36 to C36 and/or C190 to C190. Both positions 36 and 190 reside in the interior *a* heptad position, and model building leaves little doubt that formation of such a cross-link requires substantial local distortion of the coiled coil. The resulting destabilization was long ago (Lehrer, 1978) proposed to be the cause of the small, low-T transition that is seen in cross-linked αα-R-Tm via either CD or DSC (Lehrer, 1978; Privalov, 1982; Holtzer et al., 1986; Sturtevant et al., 1991). However, it has been impossible, so far, to obtain direct experimental evidence for such destabilization or to deduce its consequences definitively (Skolnick and Holtzer, 1986). In γγ-CG-Tm, the species that also has a cross-link at C190 with a local sequence rather similar to that of αα-R-Tm, the DSC curve is indeed more obviously multiphasic than that of the reduced form, but there is no neat separation into a small "pre-transition" and larger main transition, as in the case of αα-R-Tm. It is therefore not possible at present to assign any particular feature of the DSC curve in C190 cross-linked γγ-CG-Tm to such local destabilization.

Our findings thus indicate that CG-Tm homodimers unfold very much like their R-Tm counterparts, in their intermediate states; in the overall magnitudes of ΔH and ΔC_P; and, for the reduced species, in the overall dissociative nature of the transition. Apparently the mutations that distinguish these various chains lead to only small differences in thermal unfolding behavior. Although these differences may be instructive, they do not require any substantial changes in the overall unfolding model, as perhaps once seemed to be the case (Lehrer and Stafford, 1991). The appropriate model is certainly not a single two-state transition. Unfortunately, although the unfolding transition in CG-Tm as well as R-Tm homodimers is clearly more complex than the two-state, we still have no definitive way of assigning contributing subtransitions to particular local sequences. This deficiency emphasizes the need for site-specific probes in investigating unfolding transitions in coiled coils.

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