α-Helix to Random-Coil Transition of Two-Chain, Coiled Coils: Experiments on the Thermal Denaturation of Doubly Cross-Linked $\beta\beta$ Tropomyosin†

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ABSTRACT: Equilibrium thermal denaturation curves (by circular dichroism) are reported for doubly cross-linked $\beta\beta$ tropomyosin two-chain coiled coils. Cross-linking was performed by reaction of sulfhydryls with either ferricyanide or 5,5′-dithiobis(2-nitrobenzoate) (NbS_2). The extent of reaction was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and either by titration of residual sulfhydryls with NbS_2 (ferricyanide cross-linking) or by determination of mixed disulfide (protein–S–SbN) through reaction with dithiothreitol (NbS_2 cross-linking). The results indicate ~90% conversion to molecules with interchain cross-links at both C-36 and C-190. Thermal unfolding curves are compared with those obtained previously for non-cross-linked species. The curves are indistinguishable up to ~40 °C. Above ~40 °C, the doubly cross-linked species is more stable, but the transition is less steep. This relationship is also compared with that found between $\alpha\alpha$ tropomyosin (a similar coiled coil made of a genetic variant chain having a sulfhydryl only at C-190) and its singly cross-linked derivative. Thermal curves for $\alpha\alpha$ and $\beta\beta$ non-cross-linked species are very similar, $\alpha\alpha$ being somewhat more stable. For cross-linked $\alpha\alpha$, however, the curve sags at temperatures somewhat below the region of principal cooperative loss of helix, the latter occurring at higher temperature but with the same steepness as in the non-cross-linked case. The sag has been ascribed to a "pretransition" in the region of C-190. Thus, doubly and singly cross-linked species differ in that the former show no pretransition and decreased steepness in the principal transition. The significance of these findings is discussed in terms of the known physical destabilization of randomly coiled loops relative to their linear counterparts and the enormous (308-residue) loop that would exist in the completely unfolded doubly cross-linked molecule.

The muscle proteins tropomyosin, paramyosin, and the rod portion of myosin form an important structural class called coiled coils (Fraser & Mac Rae, 1973). One molecule of each comprises two right-handed α-helical chains arranged in parallel and in register and with a slight left-handed supertwist. The thermal stability of such coiled coils is of great biochemical interest, because local helix to random-coil transitions have been implicated or postulated in such diverse processes as the binding of troponin to tropomyosin (Lehrer & Betcher-Lange, 1979), the flexing of myosin as it reaches from thick to thin filaments (Pepe, 1967), and the driving of the thin filaments past the thick ones (Harrington, 1979). Moreover, this thermal stability has particular physical significance, because it may be possible to apply to this regular, repeating protein structure the physical understanding gained from study of the helix–coil transition in simpler, single-chain polypeptides (Holtzer et al., 1983; Skolnick & Holtzer, 1985). Application of these ideas in such a geometricaly well-defined molecular context may in turn have implications for the more tortuous structures in globular proteins.

Interpretations of thermal stability data on coiled coils have taken one of two approaches. In the first approach, experiments are interpreted in a physically ad hoc manner, usually in terms of putative stages of localized melting (Cohen & Szent-Gyorgyi, 1957; Noelken, 1962; Noelken & Holtzer, 1964; Woods, 1969; Halsey & Harrington, 1973; Chao & Holtzer, 1975; Lehrer, 1978; Williams & Swenson, 1981; Potekhin & Privalov, 1982; Graceffa & Lehrer, 1984). That is, it is assumed that different segments of the molecule have different inherent stability, resulting in a series of partially overlapping, essentially all-or-none, local helix–coil transitions as the temperature is raised. In the second approach, a statistical mechanical theory is used to interpret the data (Holtzer et al., 1983; Skolnick & Holtzer, 1985). This theory is an extension of the accepted theory of the helix–coil transition in single-chain polypeptides (Zimm & Bragg, 1959) and employs as a measure of the "short-range" helix-stabilizing interactions the values experimentally determined for each amino acid residue in the sequence (Scheraga, 1978). "Long-range", i.e., helix–helix, interactions are evaluated from the data. Thus, in this second approach no small subset of partially unfolded states is assumed, but rather a broad spectrum of molecular states is allowed whose relative populations are then assessed from statistical mechanics. The two approaches agree in some areas but not in others (Skolnick & Holtzer, 1985). Further work is needed to make them converge to a correct physical picture.

Both experiment and theory agree that introduction of a cross-link between helical chains can produce pronounced changes in thermal stability. This effect was first seen in skeletal tropomyosin, which is a 3 or 4 to 1 mixture of two genetic variant chains (α and β) associated into two molecular species, $\alpha\alpha$ and $\alpha\beta$ (Cummins & Perry, 1973; Eisenberg & Kielly, 1974; Yamaguchi et al., 1974). The correctness of a given picture of the transition is best tested by examining the effects of such cross-links on a single molecular species, but to date, precise data only exist on one such species of coiled coil (Holtzer et al., 1983). This molecular species is made up of a single type of chain of tropomyosin (α), which has 284 residues and a single cysteine (C-190) (Mak et al., 1979). We refer to the non-cross-linked (i.e., reduced), two-chain molecule

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as $\alpha\alpha$ and to the C-190-cross-linked molecule as $\alpha^{ss}\alpha$.[1] It is obvious that such a cross-link will influence the transition, if only because it prevents chain dissociation of the unfolded helices. Thus, the concentration dependence that characterizes the thermal transition in $\alpha\alpha$ is absent in $\alpha^{ss}\alpha$. However, recent statistical mechanical analysis predicts much deeper effects as well (Skolnick, 1985). Because of the importance of loop entropy in destabilizing any randomly coiled structure that closes on itself, the theory predicts that cross-links alter drastically the relative conformational population. The result is that thermal stability may be strongly dependent upon the number and location of such cross-links.

We report here studies of the thermal stability (by CD) of a coiled coil with two, well-separated interchain cross-links. These studies employ a second, 284-residue, genetic-variant tropomyosin chain, designated β. Each β chain has two cysteines, C-36 and C-190, the latter being in the same position as the one in α chains (Mak et al., 1979). Thermal stability studies of the reduced form, $\beta\beta$, are already extant (Isom et al., 1984). The $\beta\beta$ species denatures as $\alpha\alpha$ does, but at somewhat lower temperatures. When disulfide bonds are formed at both sites, the resulting molecule ($\beta^{ss}_{ss}\beta$) has cross-links separated by 154 residues, which in the native structure represents a linear distance of 230 Å, over half the 400-Å total length of the native molecules. In the completely unfolded state, this molecule would include an enormous, 308-residue, randomly coiled loop, a structure much less stable than its singly cross-linked counterparts. These studies should therefore be useful in further development of the physical picture of the helix–coil transition for such coiled coil proteins.

Materials and Methods

Rabbit skeletal (a mixture of $\alpha\alpha$ and $\alpha\beta$) and cardiac (pure $\alpha\alpha$) tropomyosins were prepared as described earlier (Noelken, 1962; Edwards & Sykes, 1980; Holtzer et al., 1982, 1984). Preparations of pure $\beta\beta$ tropomyosin were obtained by carboxymethylcellulose ion-exchange chromatography of reduced, denatured skeletal muscle tropomyosin and renaturation of the β fraction (Cummins & Perry, 1973). Preparations of $\alpha\alpha$ ($\beta\beta$) tropomyosin showed no signs of β (α) chains in NaDodSO$_4$/DTT/PAGE. All concentration and circular dichroism (CD) measurements were carried out in the benign medium $(NaCl)_{500}(NaP_i)_{50}(7.4)$. Circular dichroism measurements with the Jasco J-20 spectropolarimeter, temperature measurement, thermostatic control, tests for reversibility, and calculations of fraction helix from CD at 222 nm were all as previously described (Holtzer et al., 1983).

For reduced protein and for protein cross-linked with ferricyanide, absorbance at 277 nm was used to determine protein concentration. For benign media, the extinction coefficient employed was 0.314 cm^2·mg^{-1} (Holtzer et al., 1965). For protein cross-linked with NbS_2 the concentration was obtained from absorbance, corrected as follows for the presence of NbS–S–protein mixed disulfide, whose absorbance is measureable at 277 and dominant at 330 nm (Paul et al., 1980). The method requires a preliminary experiment in which $\alpha\alpha$ tropomyosin is employed. The amount of residual mixed disulfide in samples of $\alpha\alpha$ tropomyosin that had been subjected to cross-linking was determined from the fraction of non-cross-linked material, g_1, obtained from NaDodSO$_4$/PAGE. In these samples, ~10% of the molecules do not cross-link because their adjacent cysteines each form mixed disulfide. The value of g_1 is therefore the ratio C_N/C_p, wherein C_N (C_p) is the formal concentration of mixed disulfide (protein chains). The absorbance at each relevant wavelength for any such protein is given by summing over the two constituent chromophores:

$$A_{277} = C_p\epsilon_{p,277} + C_N\epsilon_{N,277} = C_p(\epsilon_{p,277} + g_1\epsilon_{N,277})$$

$$A_{330} = C_p\epsilon_{p,330} + C_N\epsilon_{N,330} = C_p(\epsilon_{p,330} + g_1\epsilon_{N,330})$$

wherein ϵ stands for molar extinction coefficient. Of the various extinction coefficients, $\epsilon_{p,277}$ = 10 400 L·mol^{-1}·cm^{-1} is immediately available from the tropomyosin value given above, with the known chain molecular mass of tropomyosin (33 kDa); $\epsilon_{p,330}$ = 472 L·mol^{-1}·cm^{-1} was readily obtained from the previous by measuring A_{330} as well as A_{277} on a stock solution of the same $\alpha\alpha$ sample before cross-linking; $\epsilon_{N,330}$ has been reported as 9170 L·mol^{-1}·cm^{-1} (Paul et al., 1980). Insertion of these values and solution of the absorbance relations for the remaining extinction give

$$\epsilon_{N,277} = [(A_{277}/A_{330})(472 + 9170g_1) - 10\,400]/g_1$$

Using the latter, our absorbance and PAGE measurements on NbS_2-cross-linked $\alpha\alpha$ protein give $\epsilon_{N,277}$ = 4990 ± 80 L·mol^{-1}·cm^{-1}. With that value, simultaneous solution of the absorbance equations for C_p gives

$$C_p = (A_{277} - 0.545A_{330})/10\,100 \text{ M}$$

or

$$C_p = (A_{277} - 0.545A_{330})/0.307 \text{ mg·cm}^{-3}$$

from which the protein concentration for NbS_2-cross-linked $\beta\beta$ protein was routinely determined from two absorbance measurements.

Cross-linking of $\beta\beta$ tropomyosin was carried out in two alternative ways by modifications of methods described by Lehrer. The first, which used $K_3Fe(CN)_6$ oxidation (Graceffa & Lehrer, 1984), was carried out as follows. The major modification is in reductions in the concentration of the oxidizing agent and in the duration of exposure of protein to it. To a solution of reduced $\beta\beta$ tropomyosin (~2.5 mg/mL) in $(NaCl)_{500}(MOPS)_5(7.5)$, sufficient additional buffer, 10 mM $K_3Fe(CN)_6$, and 0.2 mM $CuSO_4$ were added to give final concentrations of approximately 1.3 mg/mL protein, 0.08 mM sulfhydryl, 0.1 mM $K_3Fe(CN)_6$, and 2 μM $CuSO_4$. The resulting solution was stirred at room temperature for 30–40 min. Excess ferricyanide was removed either by exhaustive dialysis, first against $(NaCl)_{500}(MOPS)_5(7.5)$ and then against several changes of $(NaCl)_{500}(NaP_i)_{50}(7.4)$, or by repeated concentration and redilution in an Amicon centricon (10) microconcentrator. The second method of cross-linking, which used NbS_2 (Lehrer, 1975, 1978), was as previously described (Holtzer et al., 1984), except that excess NbS_2 was removed in the microconcentrator rather than by dialysis. The major modification to Lehrer's original method is in avoidance of high NbS_2 concentrations in order to minimize sites at which mixed disulfide forms at both sulfhydryls and thus prevents cross-linking.

[1] Abbreviations: $\alpha\alpha$ ($\beta\beta$), reduced α-tropomyosin (β-tropomyosin) dimeric (i.e., two-chain) species; $\alpha^{ss}\alpha$ ($\beta^{ss}\beta$), α-tropomyosin (β-tropomyosin) dimeric species cross-linked at C-190; $\beta_{ss}\beta$, β-tropomyosin dimeric species cross-linked at C-36; $\beta^{ss}_{ss}\beta$, β-tropomyosin dimeric species cross-linked at both C-190 and C-36; NbS_2, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism. We describe complex aqueous solvent media by giving the chemical formula or abbreviation for each solute with its millimolarity as subscript, followed by the pH in parentheses (Holtzer et al., 1965).

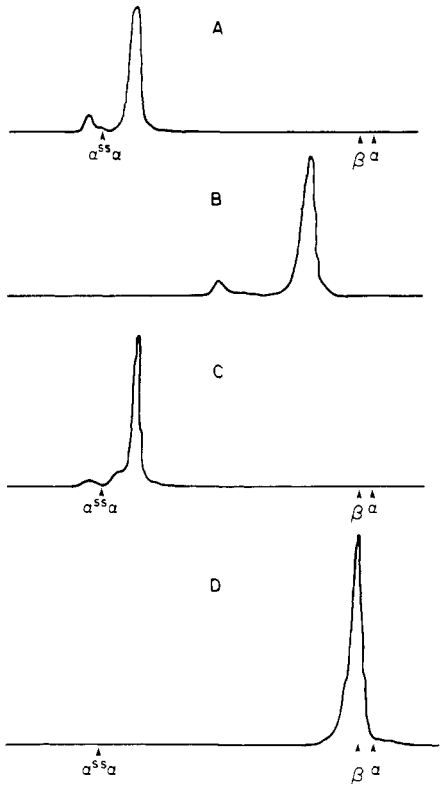

FIGURE 1: Representative scans from $NaDodSO_4$/PAGE. Electrophoresis direction is left to right. Where relevant, positions corresponding to α and β single chains and $\alpha^{ss}\alpha$ cross-linked dimers are marked by arrows. (A) $\beta\beta$ tropomyosin cross-linked with $Fe(CN)_6^{3-}$. (B) Same as (A), run twice as long. (C) Another sample similar to (A) but showing a trailing shoulder on the main band. (D) Reduced $\beta\beta$ tropomyosin showing absence of dimer and of α peaks.

After cross-linking, samples were analyzed by nonreducing $NaDodSO_4$/PAGE, with 8 or 10% Laemmli gels in long tubes (5 mm × 250 mm). The stacking gel was usually omitted since it did not improve the resolution of the bands. The staining and scanning of the gels was as previously described (Crimmins & Holtzer, 1981). Integration of the peaks was either as previously or by impressing the output voltage of the ISCO UA5 absorbance monitor onto an A/D converter (Applied Engineering Co.) followed by numerical integration on an Apple IIe computer. Aliquots of samples cross-linked by $K_3Fe(CN)_6$ were titrated with NbS_2 to determine residual free sulfhydryl as previously described (Ellman, 1959; Lehrer, 1978; Holtzer et al., 1984). Aliquots of samples cross-linked with NbS_2 were treated with NbS_2 in $NaDodSO_4$ to block residual free sulfhydryl, dialyzed to remove excess NbS_2, and then treated with DTT in $NaDodSO_4$; the amount of mixed disulfide was determined from the amount of NbS^- released.

Results and Discussion

$NaDodSO_4$/PAGE Laemmli gels on our cross-linked $\beta\beta$ tropomyosin show no material in the monomer (33-kDa) region and consistently show two bands in the dimer region (Figure 1A). There is no evidence of material of degree of aggregation greater than dimer, as is made clear when the same sample is run for a longer time (Figure 1B). The band of higher mobility is by far the more intense. Often, an additional, small, incompletely resolved trailing peak or shoulder is seen on the intense band (Figure 1C). The absence of any α chains in these preparations is shown by runs under reducing conditions (Figure 1D).

The small band of lowest mobility is resolved from the remainder. The fraction of protein in this slowest moving band varied from 5 to 10% of the total and agreed, for ferricyanide-cross-linked material, with the fraction of singly cross-linked molecules deduced from titration of residual free sulfhydryls with NbS_2. Moreover, this small band has a mobility only slightly less than $\alpha^{ss}\alpha$ tropomyosin, which is perforce only cross-linked at its single sulfhydryl-paired site (C-190). We therefore assign the small, slowest band to $\beta\beta$ molecules cross-linked only at C-190 ($\beta^{ss}\beta$) and perhaps also to molecules cross-linked only at C-36 ($\beta_{ss}\beta$) as well. Results with NbS_2-cross-linked material led to the same conclusion, with the fraction of protein in the slowest band centering nearer the upper end of the range (~10%) and in agreement with the fraction of mixed disulfide deduced from NbS^- released by DTT.

Our results thus indicate that as much as 95% of $\beta\beta$ tropomyosin is cross-linked at both C-36 and C-190 when cross-linking is performed as above with ferricyanide and only a bit less when NbS_2 is used. The mobility difference observed within the dimer region is perhaps in accord with this band assignment, since a molecule with two cross-links should be more compact and thus more mobile than the same molecule with only one cross-link (Crimmins & Holtzer, 1981; Lehrer & Joseph, 1985). The two singly cross-linked species, $\beta^{ss}\beta$ and $\beta_{ss}\beta$, are not equivalent, but they would be expected to have rather similar mobilities and probably appear in the same band. However, we cannot rule out the possibility that the small, variable band of intermediate mobility comprises species cross-linked only at C-36. Lehrer and Joseph (1985) see only the bands of highest and lowest mobilities and have assigned them simply to $\beta^{ss}_{ss}\beta$ and to both species of singly cross-linked molecules, respectively. Our assignment is thus consistent with theirs, but the question of the relative mobilities of $\beta^{ss}\beta$ and $\beta_{ss}\beta$ singly cross-linked species cannot yet be considered completely answered. This detail, however, does not affect our principal conclusion, i.e., that in our cross-linked samples the vast bulk of the molecules are doubly cross-linked. The determinations of residual sulfhydryl and DTT-released NbS^- admit of no other interpretation.

The thermal denaturation profiles of three samples of β-tropomyosin, cross-linked by two different methods and ranging from 88 to 95% doubly cross-linked, are shown as the data points in Figure 2. Also shown are spline curves for previously reported data (Isom et al., 1984) on two concentrations of reduced $\beta\beta$ tropomyosin, one higher and one lower than any of the concentrations of the cross-linked material. No systematic difference can be seen among the three samples of cross-linked protein, confirming our interpretation that the predominant species in all cases is the same. Our sulfhydryl titration and DTT-released NbS^- determinations then demand that this be the doubly cross-linked species. The absolute values obtained for the mean residue ellipticity at 5.5 °C for the three samples of cross-linked protein agree well with each other [333 ± 8 deg·cm^2·mmol^{-1} (SD)], with the average of several recent values for reduced $\beta\beta$ (336 ± 16), and with the average of many values for reduced $\beta\beta$ obtained over several years (343 ± 16). These results require that the helix content of cross-linked and non-cross-linked $\beta\beta$ be at the same very high value at low temperature. Indeed, cross-linked and non-cross-linked samples are virtually indistinguishable below 35 °C. At higher temperatures, the cross-linked protein is more stable.

Previous thermal denaturation experiments on $\alpha\alpha$, $\alpha^{ss}\alpha$, and $\beta\beta$ tropomyosins reveal certain features that now must be discussed in light of the present results on $\beta^{ss}_{ss}\beta$ tropomyosin. The observed dependence of helix content upon protein con-

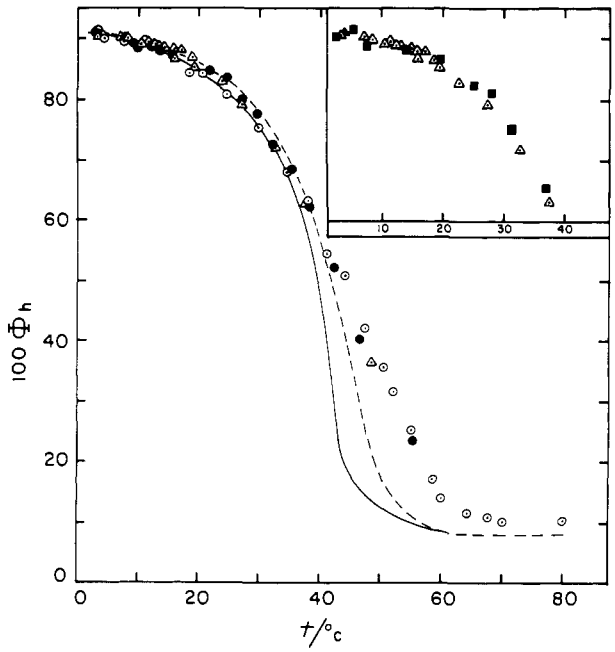

FIGURE 2: Percent helix (from CD at 222 nm) of various species of $\beta\beta$ tropomyosin vs. T. Full (dashed) curve is spline curve for non-cross-linked species at 0.100 (4.72) mg/mL from previous work (Isom et al., 1984). All data points are for doubly cross-linked ($\beta^{ss}_{ss}\beta$) species. (Open circles) 1.15 mg/mL, $Fe(CN)_6^{3-}$ cross-linked; (open triangles) 0.881 mg/mL, $Fe(CN)_6^{3-}$ cross-linked; (filled circles) 0.736 mg/mL, NbS_2 cross-linked. Inset shows experiment (see text) to double-check for pretransition. (Open triangles) Same as on main graph; (filled squares) data taken after in situ reduction of the solution that gave open triangles.

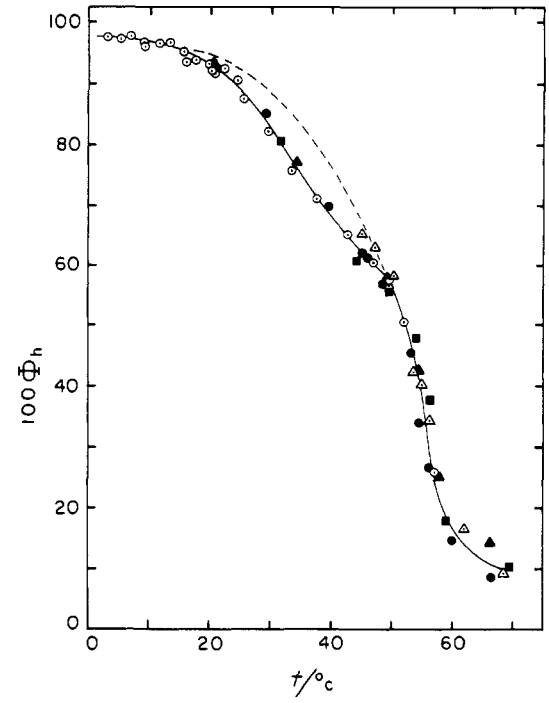

FIGURE 3: Percent helix (from CD at 222 nm) of cross-linked species of $\alpha\alpha$ tropomyosin ($\alpha^{ss}\alpha$): (full curve) spline curve through data; (dashed curve) hypothetical, "expected" segment between low-temperature (0–20 °C) and high-temperature (50–70 °C) regions; (open circles) 0.77 mg/mL; (filled squares) 0.0057 mg/mL; (filled circles) 0.057 mg/mL; filled triangles) 2.85 mg/mL; (open triangles) 8.5 mg/mL. Open circles were $Fe(CN)_6^{3-}$-cross-linked; the rest were NbS_2-cross-linked.

centration in $\alpha\alpha$ and $\beta\beta$ proteins is clearly a result of mass action since these are dissociating transitions. Such dependence is neither expected nor seen in the case of $\alpha^{ss}\alpha$ or $\beta^{ss}_{ss}\beta$. Two further features distinguish $\alpha^{ss}\alpha$ thermal profiles from those of $\alpha\alpha$. These are apparent from Figure 3, whereon are plotted our earlier data on NbS_2-cross-linked $\alpha^{ss}\alpha$ (Holtzer et al., 1983) and more recent data on ferricyanide-cross-linked $\alpha^{ss}\alpha$. The latter extend the temperature range previously available. First, either in cross-linked skeletal muscle tropomyosin (Lehrer, 1978) or in $\alpha^{ss}\alpha$ (Holtzer et al., 1983), the thermal denaturation curve has a peculiar shape in the vicinity of 35 °C. That region sags noticeably (see the solid spline curve through the data on Figure 3) compared with the shape that would be anticipated either from the curve segments at lower and higher temperature in $\alpha^{ss}\alpha$ itself or from the entire curves in $\alpha\alpha$ or $\beta\beta$ tropomyosin. This "anticipated" curve is shown as the dashed curve on figure 3.

The unusual shape in the region of 35 °C has been ascribed to a "pretransition" in the vicinity of C-190 brought about by strains in the helix induced by formation of the interchain covalent linkage (Lehrer, 1978; Holtzer et al., 1983). However, it has been pointed out that this explanation flies in the face of the pronounced *stabilizing* effect that loop entropy produces in the vicinity of such a cross-link, because a limited denatured segment in the region of such a cross-link necessarily would form a closed loop, a structure much less favored than the random coil itself (Skolnick & Holtzer, 1985). Whatever the molecular explanation, the odd shape near 35 °C cannot be an artifact of the method of cross-linking, since Figure 3 shows that data from both ferricyanide- and NbS_2-cross-linked material fall on the same curve. Moreover, it has already been shown that NbS_2- or O_2-cross-linked skeletal tropomyosin also show this effect (Lehrer, 1978). The effect is also not explainable as a result of the presence of some residual non-cross-linked $\alpha\alpha$ in the sample. There is ~10% such material in most samples, an amount easily seen to be much too small to explain the difference between the solid and dashed curves of Figure 3.

The second feature distinguishing $\alpha\alpha$ from $\alpha^{ss}\alpha$ thermal curves is the displacement of the principal cooperative transition in $\alpha^{ss}\alpha$ to higher temperature. For $\alpha^{ss}\alpha$, 50% helix is reached at 52.5 °C, whereas $\alpha\alpha$ in the same concentration range is half helix over the range 45–48 °C in the accessible concentration region. However, there is little difference in steepness of this portion of the curve; the rapidly falling region (50–70 °C) of the $\alpha^{ss}\alpha$ curve roughly parallels that for $\alpha\alpha$, merely being shifted to somewhat higher temperature.

Because of the difficulty of selective cross-linking of $\beta\beta$ molecules at C–190 *only*, no data presently exist on $\beta^{ss}\beta$ that can be compared with those for $\beta\beta$ and $\beta^{ss}_{ss}\beta$ given in Figure 2. It may be assumed, however, that such data would show a region similar to that shown by $\alpha^{ss}\alpha$, albeit perhaps displaced to lower temperature in view of the generally lower stability of $\beta\beta$ vs. $\alpha\alpha$ helices (Isom et al., 1984). The data for the doubly cross-linked species (Figure 2), however, show no such anomolous region.

To check this point more thoroughly, an experiment was done in which, after the thermal curve was run, the $\beta^{ss}_{ss}\beta$ sample was renatured, reduced by adding a carefully weighed out amount of DTT directly to the cell and allowing it to stand at 5 °C overnight, and run again. Thus, $\beta^{ss}_{ss}\beta$ and $\beta\beta$ species were directly compared under virtually identical instrumental and environmental conditions. These data are shown in the inset to Figure 2 and demonstrate that data sets for both species fall on the same curve in the relevant (low) temperature region. To verify that reduction had indeed occurred, the temperature was then raised further, yielding at 50 °C a helix content of 13.5%, clearly compatible only with $\beta\beta$ not with

$\beta^{ss}_{ss}\beta$ species. Moreover, to check the possibility that reduction did not take place *until* the sample was raised to 50 °C, it was returned to the low-temperature region and found to yield values on the original curve. We conclude that there is no pretransition in the doubly cross-linked species.

The $\beta^{ss}_{ss}\beta$ species also differ in the high-temperature region from expectations engendered by phenomenological comparison of $\alpha^{ss}\alpha$ with $\alpha\alpha$ species. The thermal curve for $\beta^{SS}_{SS}\beta$ indicates higher helix content than for $\beta\beta$, as expected. However, the steepness of the transition is appreciably less in the doubly cross-linked case than in its non-cross-linked counterpart, whereas, as already noted, the singly cross-linked species (at least in $\alpha^{ss}\alpha$) has about the same steepness as in the non-cross-linked species ($\alpha\alpha$).

These differences observed in the helix to random transition in the various species of coiled coils present a sharp challenge to physical theory. It remains to be seen whether a clear physical picture can be developed of the pronounced effects such cross-links produce in the thermal stability. Such cross-links also have pronounced effects on stability in globular proteins (Anfinsen & Scheraga, 1975; Lin et al., 1984; Skolnick, 1985). It is hoped that the simple context in which they exist in coiled coils will allow their physical effects to be more precisely defined.

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REFERENCES

Anfinsen, C., & Scheraga, H. (1975) *Adv. Protein Chem. 29*, 205.

Chao, Y.-Y., & Holtzer, A. (1975) *Biochemistry 14*, 2164–2170.

Cohen, C., & Szent-Györgyi, A. G. (1957) *J. Am. Chem. Soc. 79*, 248.

Crimmins, D., & Holtzer, A. (1981) *Biopolymers 20*, 925–950.

Cummins, P., & Perry, S. (1973) *Biochem. J. 133*, 765–777.

Edwards, B., & Sykes, B. (1980) *Biochemistry 19*, 2577–2583.

Eisenberg, E., & Kielley, W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Ellman, G. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Fraser, R., & Mac Rae, T. (1973) *Conformation in Fibrous Proteins*, Chapter 15, Academic Press, New York.

Graceffa, P., & Lehrer, S. (1984) *Biochemistry 23*, 2606–2612.

Halsey, J., & Harrington, W. (1973) *Biochemistry 12*, 693–701.

Harrington, W. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5066.

Holtzer, A., Clark, R., & Lowey, S. (1965) *Biochemistry 4*, 2401–2411.

Holtzer, M. E., Holtzer, A., & Skolnick, J. (1983) *Macromolecules 16*, 173–180.

Holtzer, M. E., Breiner, T., & Holtzer, A. (1984) *Biopolymers 23*, 1811–1833.

Isom, L., Holtzer, M., & Holtzer, A. (1984) *Macromolecules 17*, 2445–2447.

Johnson, P., & Smillie, L. (1975) *Biochem. Biophys. Res. Commun. 64*, 1316–1322.

Lehrer, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3377–3381.

Lehrer, S. S. (1978) *J. Mol. Biol. 118*, 209–226.

Lehrer, S. S., & Betcher-Lange, S. (1979) *Motility in Cell Function*, pp 381–385, Academic Press, New York.

Lehrer, S. S., & Joseph, D. (1985) *Biophys. J. 47*, 509a.

Lin, S., Konishi, Y., Denton, M., & Scheraga, H. (1984) *Biochemistry 23*, 5504–5512.

Mak, A., Lewis, W., & Smillie, L. (1979) *FEBS Lett. 105*, 232–234.

Noelken, M. (1962) Ph.D. Thesis, Washington University.

Noelken, M., & Holtzer, A. (1964) in *Biochemistry of Muscle Contraction* (Gergely, J., Ed.) pp 374–378, Little Brown, Boston.

Paul, C., Kirschner, K., & Haenisch, G. (1980) *Anal. Biochem. 101*, 442–448.

Pepe, F. (1967) *J. Mol. Biol. 27*, 203–225.

Potekhin, S., & Privalov, P. (1982) *J. Mol. Biol. 159*, 519–535.

Scheraga, H. (1978) *Pure Appl. Chem. 50*, 315–324.

Skolnick, J. (1985) *Biochem. Biophys. Res. Commun. 129*, 848–853.

Skolnick, J., & Holtzer, A. (1985) *Macromolecules 18*, 1549–1559.

Stewart, M. (1975) *FEBS Lett. 53*, 5–7.

Williams, D., & Swenson, C. (1981) *Biochemistry 20*, 3856–3864.

Woods, E. F. (1969) *Int. J. Protein Res. 1*, 29–43.

Yamaguchi, M., Greaser, M., & Cassens, R. (1974) *J. Ultrastruct. Res. 48*, 33–58.

Zimm, B., & Bragg, J. (1959) *J. Chem. Phys. 31*, 526–535.