α-Helix-to-Random-Coil Transition of Two-Chain, Coiled Coils. Theory and Experiments for Thermal Denaturation of α-Tropomyosin

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ABSTRACT: New data are presented for the α-helix content of non-cross-linked and cross-linked α-tropomyosin at near-neutral pH as a function of temperature (20-70 °C) and over a 1000-fold range of protein concentration. Helix content is obtained from circular dichroism measurements by a new method. Experimental thermal denaturation curves are dependent upon concentration for the non-cross-linked protein but independent of it for the cross-linked protein, indicating the mass action effect of chain dissociation in the non-cross-linked case. When the data are used to determine the helix-helix interaction parameter [ου(T)] introduced earlier and the resulting η(T) is used in the realized theory previously developed, a rather satisfactory fit of theory to experiment is obtained over the entire investigated range of temperature and protein concentration. The theory also generates predictions concerning the average molecular weight as a function of temperature for the non-cross-linked protein at a given concentration. The η(T) obtained for α-tropomyosin yields values for various standard thermodynamic properties (ΔG°, ΔH°, ΔS°, and ΔC°) characterizing the helix-helix interaction. Comparison is made with those obtained earlier from extant data on a short-chain synthetic model coiled coil (XY3) whose hydrophobic helix-helix interactions are entirely due to leucine-leucine contacts. The interaction free energy in XY3 is seen to be more favorable than in α-tropomyosin, largely because of its enthalpic advantage; the standard entropy of the interaction actually favors α-tropomyosin. The percent helix calculated from theory for cross-linked chains is always larger than that obtained by experiment. This disagreement is ascribed to distortions in local short-range [ε and s(T)] and/or long-range [ου(T)] interactions attendant upon formation of the disulfide link—effects not encompassed by the present theory or reflected in the parameters used to realize it. The theory is thus shown to provide a rational framework that serves to separate out effects of mass action and local distortions in comparing non-cross-linked and cross-linked proteins, since it has previously been thought that cross-linking destabilizes only part of the molecule and stabilizes the rest. Similarly, the theory is shown to allow the effects of differences in chain length, cross-linking, and helix-helix interaction to be clearly distinguished when α-tropomyosin is compared with the smaller, synthetic model coiled coil XY3.

I. Introduction

In the preceding papers in this series,1,2 a statistical theory is described that treats the thermally induced transition of α-helix to random coil for polypeptides that form two-chain α-helical, coiled coils, i.e., molecules comprising two α helices, side-by-side in parallel and in register, with a slight supertwist. The theory is couched in terms of three types of parameters, s, σ, and w, each of which embodies a particular contribution to the interactions that stabilize the helical structure. Specifically, these interactions are (1) the well-known "short-range" interactions, that lead to a free energy of initiation of a helical conformation at a particular residue [written −RT ln (σε)] and to a free energy of addition of a helical residue to an existing helical stretch [written −RT ln (s)],5-7 and (2) the "long-range" interactions, that lead to a free energy arising from the proximity of an α-helical turn on one chain to that on the second chain [written −RT ln (w)].1

Thus, of these two classes of interactions, the first corresponds to the concept of "secondary structure" and the second to the concept of "quaternary structure" as used in classical protein chemistry. Clearly, σ and s are expected to depend on the nature of the amino acid residue and w is expected to depend on the nature of the interchain pairs of residues that are in contact at the site in question; thus, both classes reflect the primary structure of the chain. Moreover, s and w are expected to be temperature dependent. In practical applications, the theory can be "realized" by use of the extensive determinations of ε and s(T) for the relevant amino acids from Scheraga's laboratory.8-23 Use of these values by Mattice et al.24-25 to calculate properties of single, partially α-helical protein chains (where w(T) is not required) has led to very promising results. Recently, the entire collection of values of the s and s(T) parameters has been summarized in convenient algorithmic form.2 Recent modifications (for Ile and Gln) are described below.

Tropomyosin is the prototypical α-helical, two-chain, coiled coil and, moreover, is a protein important in muscle contraction.26-40 Each of the polypeptide chains in the molecule has 284 residues. In the form isolated from rabbit cardiac muscle, the chains (designated "α chains") have the same, known sequence. Obviously, this is the most attractive object to which the two-chain theory could be applied. However, since detailed information on w(T) for the various types of interchain contacts is not available, the first application of the theory2 was made to a synthetic polypeptide, designated XY3, that has a 43-residue chain of a sequence much more uniform than tropomyosin's but that was carefully chosen by its creators, Hodges and co-workers, to mimic the pattern of hydrophobic and charged residues in the sequence of tropomyosin.41 This synthetic material had been found to display the α-helical, two-chain, coiled-coil structure and could be studied with the chains either cross-linked (by a single disulfide link near one end) or not cross-linked. Such a chain is expected to display little variation in w(T) along its length, and the theory, with an appropriate choice of w(T), was found to be capable of fitting the thermal denaturation data for the cross-linked molecule.4 Only very limited data for the non-cross-linked molecule are as yet available for this synthetic substance.

Application of the realized theory to tropomyosin was necessarily tentative for several reasons. There is the
question of local variations in \( w(T) \) along the chain, already described above. However, even if one is prepared to accept use of an appropriate average \( w(T) \) at every point, there are still difficulties. Most extant data on thermal transitions of tropomyosin were taken for protein isolated from skeletal muscle, which is a natural (~4:1) mixture of two types of chains (\( \alpha \) and \( \beta \)) differing somewhat in amino acid composition and sequence. The effects of such heterogeneity may be material. Furthermore, theory demands that separated chains of tropomyosin have rather low helix content, the high helix content observed at near room temperature being, perforce, attributed to helix-helix attractive interactions. Thus, non-cross-linked chains should dissociate upon destruction of the helical structure and, concomitantly, of the chain-chain attraction. If so, then theory, as well as the most elementary mass actional considerations, also demands that the thermal denaturation curves be dependent upon protein concentration. Yet, although tropomyosin denaturation curves have been under observation for some years in several laboratories (including this one), protein concentration has hitherto been considered so minor in interest that publications often do not even contain a record of it. Finally, although the latter difficulty seemingly can be circumvented by careful study of the cross-linked protein (which cannot dissociate), the only such study extant, that for Lehrer's laboratory, concerns skeletal (and therefore heterogeneous) tropomyosin and, furthermore, reveals a complex denaturation curve characterized by an anomalous "pretransition" that is difficult enough to interpret qualitatively and complicates quantitative interpretation enormously.

For these reasons we report here an experimental investigation of the circular dichroism of non-cross-linked and cross-linked rabbit cardiac tropomyosin (which comprises only \( \alpha \) chains) as a function of temperature and protein concentration in the region of near-neutral pH. In this study, we confine attention to the region of the principal transition (20–70 °C). The statistical theory under discussion deals with the helix content of the solution and not circular dichroism, per se. Consequently, it is necessary to obtain as good a measure as possible of the helix content. We have therefore also reexamining the means of calculating helix content from circular dichroism data and report here on what we hope is an improved method. Finally, we describe our efforts to fit the realized theory to the data and the light the results cast on the experimental findings.

II. Methods

Protein Preparation and Manipulations. Rabbit cardiac tropomyosin was prepared by minor modification of standard procedures comprising preparation of an alcohol-acetone powder, extraction, and several successive ionic strength and ammonium sulfate precipitations. The protein solution was lyophilized after each extraction plus washes, and the resulting preparation was judged satisfactory by the \( A_{280} / A_{280} \) ratio, polyacrylamide gel electrophoresis in sodium dodecyl sulfate under non-reducing conditions; gel scans showed it to be 93% cross-linked.

Cross-linking of the \( \alpha \)-tropomyosin chains was carried out using \( \text{Nbs}_2 \) according to a modification of the procedure of Lehrer. To an ~8 mg mL\(^{-1} \) solution of the protein in \((\text{NaCl})_{500}(\text{NaPi})_{50}(7.4)\) was added over a 24-h period four aliquots of \((\text{NaCl})_{500}(\text{NaPi})_{50}(7.4)\) of volume such that each contained a 3:1 molar ratio of \( \text{Nbs}_2 \) to tropomyosin dimer. The excess \( \text{Nbs}_2 \) was then dialyzed out. The preparation was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate under nonreducing conditions; gel scans showed it to be 93% cross-linked.

Circular Dichroism and Temperature Measurements. A Jasco Model J-20 was employed to measure CD. There is some disagreement in the literature concerning absolute calibration of CD instruments. Some favor calibration with \( 2\text{-}p\)-antipantoic acid and others favor \( 4\text{-}10\text{-}camphorsulfonic acid; nor is there universal agreement on the precise procedures by which the calibration is to be effected or on the precise absolute value to be assigned and preparative procedure adopted for a given compound. We have found, for example, that calibration with \( 2\text{-}p\)-antipantoic acid (TRI-DOM Chemical) in water (30.0 mM in a 0.0100-cm cell to 0.800 mM in a 1.00-cm cell) at room temperature using for the peak at 219 nm \( [\theta]_{219} = -173 \text{deg cm}^2 \text{mmol}^{-1} \) gives values for the mean residue ellipticity of tropomyosin when it is very highly helical (e.g., near-neutral pH, 25 °C) of ~400 deg cm\(^2\) mmol\(^{-1}\) at 222 nm. Using a different calibration method, Yang et al. found for rabbit skeletal tropomyosin at 222 nm, near-neutral pH, and 25 °C \( [\theta]_{222} = -350 \) in the same units. Since our calculation of fraction helix from mean residue ellipticity depends mainly on work from Yang's laboratory, we elected to adjust our absolute calibration to conform with theirs.

Because of the need to control temperature and to cover a wide range in protein concentration, several jacketed cells (Hillers) were used with path lengths of 0.0100, 0.500, 1.00, and 5.00 cm. Cell-to-cell agreement for the same solution or for solutions quantitatively diluted from a stock was quantitative for solutions under conditions such that mean residual ellipticity is expected to be independent of concentration.

Temperature regulation in the jacketed cells was accomplished by circulating ethylene glycol from a Polysciences Model 90 low-temperature bath and circulator. Temperature measurements were made with an Omega Engineering, Inc., Model 450 digital thermometer equipped with a subminiature thermocouple (chromel–constantan) probe. The temperature of the solution in the illuminated volume inside the 0.5-, 1.0-, and 5.0-cm cells was measured at various bath temperatures and plotted vs. the bath temperature. Within the precision of the temperature measurement, the points for all three cells lie on the same straight line: root-mean-square residual = 0.3 °C.

Since even this small probe is too large to fit between the internal optical faces of the 0.01-cm cell, the temperature was measured with the probe immersed in the solution inside the cell just adjacent to the very narrow region through which the light beam passes and also with the probe touching the exterior of the cell faces, which are indented in these cells so that they are air-thermostated by the jackets. When plotted vs. the bath temperature, these points fall on the same line as those for the other three cells. Thus, the temperature inside the 0.01-cm cell is taken to be the same as the temperature inside the other cells at the same bath temperature.

In any case since all measurements were at or above ambient temperature, the temperature inside the 0.01-cm cell cannot be lower than the temperature of the cell face or higher than the bath temperature. If the temperature inside this cell is, in fact, higher (as that given by the temperature curve which is the same as the temperature of the cell face), the only effect would be to shift the denaturation profiles for the highest concentration to slightly higher temperatures (maximum of 1.5 °C at 70 °C). Such a shift would not be great enough to change any of the conclusions drawn; indeed, it would improve the fit to theory.

After each temperature change, approximately 10 min was allowed before the spectra were recorded. Then, two to four scans were recorded at each temperature; these showed no systematic
variation with time, indicating that temperature had equilibrated. In recording all denaturation profiles, temperatures were decreased as well as increased, especially in the transition region. In all studies involving reduced protein, the temperature was decreased slowly from the highest temperature to about 38 °C, where cross-linked and un-cross-linked samples have quite different helix content, and another set of scans taken to ensure that the protein had remained reduced throughout. In some cases, the entire denaturation profile was retracted. We have no doubt that the CD measurements are of equilibrium states.

Solvent base lines were run with the same sensitivity scales and temperature ranges as for the solutions.

**Calculation of Fraction Helix from CD.** To obtain the fraction of residues in helical form from the observed mean residue ellipticity of the solution, \([\theta]\), it is necessary to assess the mean residue ellipticity for a complete helical chain and for a completely randomly coiled chain. Yang and co-workers have supplied important experimental information toward this end.\(^5\) They find that the CD of a fully \(\alpha\)-helical chain results from contributions of three spectral bands and that the intensities depend upon the number of residues in the chain \(n\). They give the mean residue ellipticity at wavelength \(\lambda\) of a complete \(\alpha\) helix of \(n\) residues in the form\(^5\)

\[
[\theta_n^H] = -386[1 - 2.55n^{-1}] + [\theta_0]^H[1 - 2.55n^{-1}]
\]

In the following, we will employ eq 2 to obtain the contribution of a helical stretch of \(n\) residues to the observed mean residue ellipticity at 222 nm.

The value appropriate to a complete random coil is somewhat less certain. In earlier work, the value \([\theta_0]^H = +15.80\) degn\cdot cm\(^2\)\cdot mol\(^{-1}\) at 222 nm seemed the best choice,\(^5\) some workers on tropomyosin have employed that figure. More recently,\(^5\) however, Yang et al. favor \([\theta_0]^H \approx -20.00\) degn\cdot cm\(^2\)\cdot mol\(^{-1}\). Experience in our own laboratory with thermal and guanidinium chloride induced denaturation of tropomyosin and paramyosin suggests \([\theta_0]^H \approx 0.0\) because of this uncertainty, we will retain the symbol \([\theta_0]^H\) in the development that follows. In practice, we have made actual calculations using both \([\theta_0]^H = -20.00\) and \([\theta_0]^H = 0.0\); values for fraction helix reported in this paper were obtained by averaging the resulting two values of fraction helix. The two values averaged do not differ very much except at rather small helix content, where this difficulty becomes the principal source of uncertainty in the calculation. We next apply these ideas to the case of a partially denatured system in which only helical regions and random regions coexist. This presumably applies to tropomyosin.

In such cases, as Yang et al. have shown, the mean residue ellipticity is given by\(^5\)

\[
[\theta] = [\theta_n^H]\Phi_h + [\theta_0]^H\Phi_c = [\theta_n^H]\Phi_h + [\theta_0]^H(1 - \Phi_h)
\]

in which \(\Phi_h\) (\(\Phi_c\)) is the fraction helix (random). \([\theta_n^H]\) is the mean residue ellipticity for a helical residue located within a helical stretch \(n\) residues in length, \(n\) is the average length, in number of residues, of a helical stretch in the molecule, and \([\theta_0]^H\) is the mean residue ellipticity of a random residue.

Substitution of eq 2 into eq 3 gives after rearrangement

\[
[\theta] - [\theta_0]^H = ([\theta_n^H] - [\theta_0]^H)\Phi_h - (2.55[\theta_n^H]/\Phi_h)
\]

As it stands, eq 4 cannot be directly implemented to allow computation of \(\Phi_h\) from \([\theta]\), because although \([\theta_n^H]\) and \([\theta_0]^H\) can be estimated as described above, we do not, in general, know \(n\). Somehow, we must estimate that quantity to implement eq 4.

To estimate \(n\), we first define a quantity \(I\) as the average number of helical regions in a molecule. We then can write

\[
n/I = n/\Phi_h
\]
so no change whatever is needed in results previously reported for it or its homologues.

To fit experimental thermal denaturation data to the theory, the following procedure was used, which differs somewhat from that used earlier. The mean residue ellipticity at 222 nm at each temperature was converted to fraction helix as described above. A plot of $\phi_s$ vs. temperature was made and a smooth curve drawn through the experimental points by eye. Fits to the theory were always made by using "smoothed experimental points", i.e., points picked off the smooth curve through the data. This has several advantages. It legitimately smooths the unavoidable experimental scatter; it allows integral temperatures to be used, which speeds data entry for the computations; and it allows additional points to be employed after the fact, if a particular region turns up results unforeseen.

Each smoothed experimental point is then compared with theoretical curves (generated for the same temperature and protein concentration) of $\phi_s$ vs. $w$ to estimate the value of $w$ required to obtain from theory the smoothed experimental value of $\phi_s$. Once $w$ has been estimated, a separate equation is used with trial values in the vicinity of the estimate to obtain the exact value of $w$ required to produce the smoothed experimental value of $\phi_s$.

Once this has been done for all experimental points, i.e., for a series of temperatures and protein concentrations, all the data are plotted as $RT \ln w$ vs. $T$. The next step is to obtain a function that best fits these data. Earlier, this was accomplished by computer fitting all the points to a second-order polynomial. In the present work, a somewhat different procedure was employed. Points were included in the fit only if 0.15 $\phi_s < 0.95$. The reason for this lies in the nature of the functional dependence of $\phi_s$ on $w$. Outside the range indicated, $\phi_s$ becomes extremely insensitive to $w$, so that small scatter in $\phi_s$ values is magnified into catastrophic excursions in $w$. The smoothed data in the included range were then computer fit to the equation

$$-\Delta G^o = RT \ln w = BT \ln T + A_0 + A_1 T + A_2 T^2$$  \hspace{1cm} \hspace{1cm} (7)$$

wherein $B$, $A_0$, $A_1$, and $A_2$ are constants and $T$ is the Kelvin temperature. The reason for choice of this representation of the negative of the standard free energy of interaction is that it then provides functional forms for the other thermodynamic properties that are more in keeping with the way they are ordinarily represented; i.e., $\Delta H^o$ and $\Delta C_p^o$ emerge as polynomial expansions:

$$\Delta S^o = B \ln T + B + A_1 + 2A_2 T$$  \hspace{1cm} \hspace{1cm} (8a)$$

$$\Delta H^o = -A_0 + BT + A_2 T^2$$  \hspace{1cm} \hspace{1cm} (8b)$$

$$\Delta C_p^o = B + 2A_2 T$$  \hspace{1cm} \hspace{1cm} (8c)$$

Although this form is different from that previously employed, the numerical results are materially changed. We refit the $RT \ln w$ values for X-Y$_5$, for example, and find essentially the same numerical values for the calculated thermodynamic parameters.

Equation 7 with the computer fitted values of the coefficients can then be used to obtain calculated ("theoretical") values of $w(T)$ and the now fully realized theory used to obtain overall fraction helix, fraction of dimer, dimer helix content, and other relevant properties of the system.\textsuperscript{1,2}

III. Experimental Results

Non-Cross-Linked $\alpha$-Tropomyosin. Experimental denaturation curves for non-cross-linked (reduced) $\alpha$-tropomyosin are shown in Figure 2 as percent helix vs. Celsius temperature for the extreme protein concentrations, 0.0044 and 5.2 mg-mL$^{-1}$. The points shown are actual experimental values calculated as described above from the measured mean residue ellipticities. It is clear from the figure that the denaturation curve of non-cross-linked tropomyosin is concentration dependent, but it is also clear that this would easily be missed without careful temperature control and measurement and coverage of a rather sizable concentration (over 1000-fold) range. Figure 2 gives some idea of the experimental scatter. The curves through all such data for each concentration were drawn by eye in the absence of data for the other concentrations. The resulting "eyeballed" curves are also shown in Figure 2 and inasmuch as points picked off these curves ("smoothed experimental points") were used to obtain theoretical fits, it is important for the reader to judge from Figure 2 how fairly the drawn curves represent the actual data points.

Cross-Linked $\alpha$-Tropomyosin. Experimental denaturation experiments for cross-linked $\alpha$-tropomyosin are shown in Figure 3 in a similar manner. Here, experimental data (squares) cover an even wider range of protein concentration (0.0055-8.2 mg-mL$^{-1}$), yet all such data fall on a single (solid) curve. This lack of concentration dependence is in clear contrast to our findings for the non-cross-linked protein.

To aid comparison of the shapes of the temperature profiles for cross-linked and non-cross-linked material, thermal denaturation data for an intermediate concentration (0.104 mg-mL$^{-1}$) of the non-cross-linked protein are also shown in Figure 3. Clearly, the profile of the cross-linked material shows the peculiar shape described earlier

![Figure 2](image_url)  \hspace{1cm} \hspace{1cm} Figure 2. Observed percent helix vs. Celsius temperature for non-cross-linked $\alpha$-tropomyosin. Open circles and full curve: protein at 0.0044 mg-mL$^{-1}$ in 5.00-cm cell; filled circles and full curve: protein at 5.2 mg-mL$^{-1}$ in 0.01-cm cell. Medium in both cases is (KCl)$_{500}$ [KF]$_{100}$ (DTT)$_{1.7}$ (7.4). Each curve drawn by eye to fit data.

![Figure 3](image_url)  \hspace{1cm} \hspace{1cm} Figure 3. Experimental percent helix vs. Celsius temperature for cross-linked and non-cross-linked $\alpha$-tropomyosin. Bottom-filled circles and dashed curve: non-cross-linked protein at 0.104 mg-mL$^{-1}$ in 0.500-cm cell. Squares and full curve: cross-linked protein at 0.0055 mg-mL$^{-1}$ in 5.00-cm cell (open); 0.055 mg-mL$^{-1}$ in 1.000-cm cell (bottom filled); 2.6 mg-mL$^{-1}$ in 0.01-cm cell (top filled); 8.2 mg-mL$^{-1}$ in 0.01-cm cell (filled). Medium in all cases is (KCl)$_{500}$ [KF]$_{100}$ (7.4), with non-cross-linked protein also containing 1 mM DTT. Each curve drawn by eye to fit data.
by Lehrer in his study of the skeletal muscle protein and ascribed to a "pretransition". The reader will also verify that the experiment for non-cross-linked material of intermediate concentration (Figure 3) occupies a position between those at the two extreme concentrations shown above (Figure 2).

IV. Theoretical Results

Non-Cross-Linked \( \alpha \)-Tropomyosin. At least a dozen points were picked off each denaturation curve shown in Figures 2 and 3 and the value of \( w \) consistent with each point at the appropriate concentration and temperature was derived from the realized theory. The resulting array of points is shown in Figure 4 as \( RT \ln w \) vs. Celsius temperature. As seen, although perhaps some small dependence upon concentration exists, the general pattern is approximately parabolic with a minimum near 40 °C. Flagged points indicate those with \( \Phi_h \) values outside the range deemed reliable for determination of \( w \) (see above) and were omitted from the computer fit to the data. The unflagged data (27 points) are seen to follow rather well this computer fit (solid curve), which is given by eq 7 with \( A_0 = 25.402.9886, A_1 = -157.723.822, A_2 = 0.253.298.173, \) and \( B = -0.178.481.715 \). The resulting equation, which shows a minimum at 41.5 °C, can then be used with the realized theory to calculate various properties of the system for a variety of chosen concentrations and temperatures.

How well the fit \( w(T) \) represents the experiments can be seen from Figure 5, wherein are plotted smoothed points from experimental curves of Figure 2 along with theoretical curves generated by using the \( w(T) \) with the realized theory. The theoretical curve for noninteracting (\( w = 1 \)) helices of \( \alpha \)-tropomyosin is also shown (dashed) for comparison. Figure 5 indicates that the fully realized theory is rather satisfactory, although it perhaps slightly overestimates the effect of changing concentration, tending to produce at a given temperature somewhat smaller values of \( \Phi_h \) than observed at the lowest concentration and somewhat larger ones at the highest concentration. As would be anticipated from Figure 5, intermediate concentrations (not shown) agree virtually exactly with theory. We believe it fair to say that the fully realized theory is quite consistent with the observed percent helix of non-

Cross-Linked \( \alpha \)-Tropomyosin. Figure 6 shows smoothed experimental points (squares) obtained from our data on cross-linked protein (see Figure 3). The fully realized theory allows calculation of the percent helix of the dimer species at a given temperature. This quantity is, of course, independent of concentration and in the cross-linked case is also the percent helix of the whole solution since only dimer species are present. That theoretical curve is also shown in Figure 6 (solid curve), from which it is clear that theory and experiment disagree markedly, the theory requiring a helix content appreciably larger than the experiments display over virtually the entire temperature range.

V. Discussion

Non-Cross-Linked \( \alpha \)-Tropomyosin. The concentration dependence shown by the experiments (Figure 2) clearly indicates a mass actional effect on the thermal...
transition in this system. It thus seems inescapable that the principal thermal transition in α-tropomyosin is accompanied by chain dissociation. This was previously explicitly suggested and has probably been tacit in the work of several laboratories for some time but has not, to our knowledge, hitherto been demonstrated experimentally. In the different case of denaturation by guanidinium chloride, dissociation has been demonstrated, but by direct measurement of molecular weight by sedimentation equilibrium.\(^{(56)}\) Once one is aware that a very large concentration range is required, it becomes evident that CD itself suffices to demonstrate the presence or absence of chain dissociation. The appropriateness of the method is reinforced by the finding that the cross-linked protein shows no such concentration dependence. Since such a conclusion concerning chain dissociation depends only upon the law of mass action, its validity clearly is independent of any specific theory of chain–chain interaction.

Since the fully realized theory under scrutiny fits the experimental data of helix content vs. temperature over a wide range of concentration, we tentatively conclude that, in the absence of interactions, the theory predicts that, in the absence of interactions, the theory predicts that the principal thermal transition in α-tropomyosin is accompanied by chain dissociation. This was previously explicitly suggested and has probably been tacit in the work of several laboratories for some time but has not, to our knowledge, hitherto been demonstrated experimentally. In the different case of denaturation by guanidinium chloride, dissociation has been demonstrated, but by direct measurement of molecular weight by sedimentation equilibrium.\(^{(56)}\) Once one is aware that a very large concentration range is required, it becomes evident that CD itself suffices to demonstrate the presence or absence of chain dissociation. The appropriateness of the method is reinforced by the finding that the cross-linked protein shows no such concentration dependence. Since such a conclusion concerning chain dissociation depends only upon the law of mass action, its validity clearly is independent of any specific theory of chain–chain interaction.

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an array of speaking, the helix content of two tropomyosin chains from experiments on non-cross-linked α-tropomyosin. Hence, the solid curve on Figure 6 records, strictly polypeptides and on a helix interaction, \( w(T) \), deduced here under the theory relies for its realization upon thermodynamic quantities (Figure 8) are qualitatively comparable to those found earlier and the difficulties in reconciling such dependence with the putative hydrophobic and electrostatic molecular origins of the underlying interactions have already been sufficiently stressed. It is perhaps significant that Figure 8 indicates that the net free energetic advantage in interhelix stabilization possessed by the model XY₅ over α-tropomyosin is entirely enthalpic in nature, overriding an entropic contribution that actually favors the protein.

Cross-Linked α-Tropomyosin. We have already noted that the lack of concentration dependence seen in the cross-linked case (Figure 3) is in accord with the impossibility of dissociation. We have also remarked on the thorough disagreement (Figure 6) between the measured helix content and that given for dimeric species by the fully cross-linked case (Figure 5). The discrepancy is disturbing but perhaps understandable. The theory relies for its realization upon an array of \( s \) and \( \sigma \) values derived for each amino acid residue from experiments on non-cross-linked synthetic polypeptides and on a helix interaction, \( w(T) \), deduced here from experiments on non-cross-linked α-tropomyosin. Hence, the solid curve on Figure 6 records, strictly speaking, the helix content of two tropomyosin chains whose short- and long-range interactions are identical with those of the non-cross-linked dimer, but which are tethered at one point so they cannot undergo independent translational motion throughout the solution. That is, we are imagining a cross-link that has no effect other than "stapling" the chains together at one point. The solid curve on Figure 6 records the behavior required by the theory of such a "stapled dimer". The experiments on Figure 6, unfortunately, record the behavior of a real dimer, cross-linked, perforce, by a real disulfide bond at Cys-190. We are forced to the view, by the disagreement manifest on Figure 6, that constraints attendant upon formation of the disulfide bridge between chains so alter the \( s(T) \) and \( \sigma \) values in the vicinity of Cys-190, and possibly the interhelix interaction \( w(T) \) as well, as to render meaningless the realization of the theory presently accessible to us. We wish to point out that this conclusion is a restatement in more physical language of a position already clearly presented by Lehrer, who was the first to observe and attempt to explain the peculiar denaturation curve of cross-linked (skeletal) tropomyosin.42

We can actually learn something from this complication. Note from Figure 6 that the putative stapled dimer is more stable than the actual cross-linked molecule over essentially the entire accessible temperature range, indicating that the constraints imposed by disulfide formation reduce considerably the stability of the double-helical structure; this effect is somewhat concealed in customary direct comparisons of cross-linked with non-cross-linked protein because of the mass actionional drive toward denaturation in the latter, which is totally absence in the former. Such direct comparisons lead to the peculiarly unsatisfactory view that the cross-link stabilizes the double helix in some places and destabilizes it in others, a view that seems erroneous in the light of the present theory. It is worth emphasizing that only the existence of an explicit theory allows us to make the necessary comparisons because it allows us to deduce from the non-cross-linked case how an undistorted (stapled) dimer would behave. This need for physical theory is independent of whether the one here expounded turns out to be true or false.

Recently, Mattice and Skolnick48 have developed a theory of the helix–coil transition in cross-linked, two-chain, α-helical coiled coils that explicitly considers the detailed effects of cross-linking on the helix probability profiles and overall helix content. In addition to the helix–helix interaction parameter \( w \), they introduce a second parameter \( \omega \) that accounts for modifications in \( \sigma \) and \( s \) of the amino acid residues at the cross-linked site. For values of \( \omega < 1 \), the qualitative behavior of the thermal denaturation profiles calculated in ref 58 is the same as that of the experiments on cross-linked tropomyosin shown in Figure 6. Work is under way to apply this more detailed theory of cross-linked chains to these data.

Since the stabilization free energy for the XY₅ model substance was obtained from data on cross-linked XY₅, one might well be suspicious that similar constraints are having a distorting effect and call into question the values of \( -\Delta G_{\text{XV}} \) previously obtained. We do not believe this to be the case. In the XY₅ molecule, as has already been stated, the cross-link is very near the end of the chain, which is almost always randomly coiled anyway. Consequently, we do not believe that alterations of \( \sigma, s(T) \), or \( w(T) \) in its vicinity can have much effect on the overall helix content. If there is any residual effect, this can serve only to increase the difference seen in Figure 8 between the interchain stabilization free energies of XY₅ and of α-tropomyosin. This question could be resolved un-
equivocally by thermal denaturation studies on the non-cross-linked XY₅ at known concentration in the absence of urea, but such studies do not yet exist and, since XY₅ is not easily synthesized, they may not soon be carried out. Acceptance of the stabilization free energy previously obtained for XY₅ and its homologues implies acceptance of the calculated fraction helix for its stapled dimers, as previously obtained. The resulting curves are also shown on Figure 6 for XY₅ (dashed) and XY₃₉ (dot-dash), the latter being the homologue of XY₅ with a 281-residue chain length, close to that of α-tropomyosin. It is remarkable that the behavior of the dimeric tropomyosin model XY₅ is not terribly different from that calculated for the undistorted, dimeric species of α-tropomyosin. According to the present theory, this similarity is a fortuitous result of cancellation of the effect of chain length (which favors tropomyosin) and of interhelix stabilization (which favors XY₅), the more proper comparison being between stapled XY₃₉ dimers and stapled α-tropomyosin dimers, a comparison which clearly displays the increased interhelix stabilization of the leucine–leucine core in XY₃₉ over that in tropomyosin. This example serves to emphasize again in tropomyosin. This example serves to emphasize again in part by the Petroleum Research Fund, administered by the American Chemical Society. We thank Prof. J.-T. Yang for an informative discussion concerning determination of helix content from CD and for reading a pre-