Preferential Assembly of the Tropomyosin Heterodimer: Equilibrium Studies†

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ABSTRACT: Thermal unfolding/refolding studies of the three tropomyosin dimers, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, from chicken gizzard muscle were performed to explain the preferential assembly of α- and β-tropomyosin subunits into heterodimers, $\alpha\beta$ [Lehrer, S. S., & Qian, Y. (1989) *J. Biol. Chem. 265*, 1134]. Circular dichroism measurements showed that all three dimers unfolded in cooperative reversible transitions with $T_{1/2}$ = 40.0 °C and $\Delta H°$ = 162 kcal/mol for $\alpha\alpha$ and with $T_{1/2}$ = 42.6 °C and $\Delta H°$ = 98 kcal/mol for $\beta\beta$ at 0.4–0.5 μM concentrations. Fluorescence measurements on pyrenyliodoacetamide-labeled tropomyosin showed that (i) excimer fluorescence decreases in parallel with unfolding of homodimers, (ii) at physiological temperature, heterodimers are formed from micromolar mixtures of homodimers over a period of minutes, and (iii) heterodimers unfold/refold with temperature without appreciable formation of homodimers. To understand the preferential formation of $\alpha\beta$, we calculated the concentrations of all species present as a function of temperature for equal total amounts of α and β, using the measured thermodynamic constants of the unfolding/dissociation equilibria for $\alpha\alpha$ and $\beta\beta$. Values for $\Delta H°$ = 225 kcal/mol and $T_{1/2}$ = 43 °C for unfolding of $\alpha\beta$ at 0.5 μM concentration were obtained from the best fit of the calculations to the measured helical content vs temperature of $\alpha\beta$. The calculations confirmed that in mixtures at physiological temperature and below, $\alpha\beta$ is preferentially assembled, as a consequence of $\Delta G°_{\alpha\beta} \gg [1/2(\Delta G°_{\alpha\alpha} + \Delta G°_{\beta\beta})]$ where the $\Delta G°$'s are the standard free energies of dissociation of the respective dimers. An explanation is also offered for the formation of homodimers rather than heterodimers when assembly takes place at low temperatures from high concentrations of unfolded α and β subunits.

A number of recent studies indicate that heterodimers are preferentially assembled for the coiled-coil proteins tropomyosin (Bronson & Schachat, 1982; Lehrer et al., 1989; Sanders et al., 1986) and keratin (Coulombe & Fuchs, 1990; Hatzfeld & Weber, 1990; Steinert, 1990), and a class of DNA-binding proteins containing a coiled-coil domain (O'Shea et al., 1989). We have recently shown that for tropomyosin purified from two different muscles, the heterodimer is formed via subunit exchange during incubation of a mixture of homodimers at physiological temperature, i.e., $\alpha\alpha + \beta\beta \rightarrow 2\alpha\beta$ (Lehrer & Qian, 1990; Lehrer et al., 1989). Subunit exchange thus provides a simple mechanism to explain why the heterodimer is the predominent dimer if $\alpha\alpha$ and $\beta\beta$ homodimers are separately biosynthesized. We suggested that in a mixture of subunits at temperatures where one of the homodimers is unstable, the more stable heterodimer forms; i.e., the heterodimer is the preferred equilibrium species (Lehrer & Qian, 1990; Lehrer et al., 1989). An analogous explanation was independently offered for the preferential formation of Fos and Jun heterodimeric "leucine zipper" coiled-coils (O'Shea et al., 1989).

In the present work, we measured the thermal unfolding profiles of the three dimer species of gizzard tropomyosin (GTm): $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$. We obtained the thermodynamic constants associated with the observed cooperative transitions of the homodimers with the assumption that dissociation occurs in parallel with unfolding. GTm was chosen as a model system because circular dichroism studies have indicated the presence of only a single unfolding transition (Woods, 1976; Lehrer et al., 1984; Sanders et al., 1986), in contrast to striated muscle tropomyosin where at least two thermal unfolding transitions are apparent (Woods, 1976; Betteridge & Lehrer, 1983; Potekhin & Privalov, 1982). With the thermodynamic constants obtained for each homodimer and the equilibria involved for equal total amounts of subunits using the experimental reversible helix unfolding profile of $\alpha\beta$, we calculated the concentration of all of the associated molecules, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, and dissociated subunits, α and β, as a function of temperature. The calculated results show that the $\alpha\beta$ dimer is preferentially formed at temperatures below its unfolding transition; at higher temperatures, $\alpha\beta$ unfolds the dissociates to $\alpha + \beta$ subunits with less than 10% re-formation of either homodimer. With the use of pyrene-labeled homodimers of GTm, whose excimer fluorescence is abolished on dissociation into isolated subunits (Betcher-Lange & Lehrer, 1978; Burtnick et al., 1988), we verified that subunit exchange occurs at physiological temperature, forming the pyrene-$\alpha\beta$ heterodimer which then unfolds and dissociates at higher temperatures without appreciable re-formation of homodimers.

Experimental Procedures

Native smooth muscle GTm, which consists of a 1:1 mixture of α and β subunits assembled as $\alpha\beta$, was prepared from acetone powder of chicken gizzard at temperatures below 5 °C (Lehrer et al., 1984). The α and β subunits were separated on a preparative Vydac C18 reversed-phase HPLC column operating at 51.5 °C and the fractions were pooled as indicated (Figure 1), concentrated by flash evaporation, and lyophilized. The yield was about 1.8 mg of each subunit, identified with SDS-PAGE, from about 3.9 mg of $\alpha\beta$ loaded on the column. The α and β subunits were renatured to form $\alpha\alpha$ and $\beta\beta$ by incubation in 5 M GdmCl, 1 mM EDTA, and 5 mM dithiothreitol in Tris-HCl buffer, pH 7.5, for 0.5 h and dialysis vs 0.1 M NaCl/1 mM EDTA in 10 mM sodium phosphate buffer, pH 7.0. Native GTm was used as the $\alpha\beta$ dimer. The dimer concentration of $\alpha\beta$ was calculated by using ΔA =

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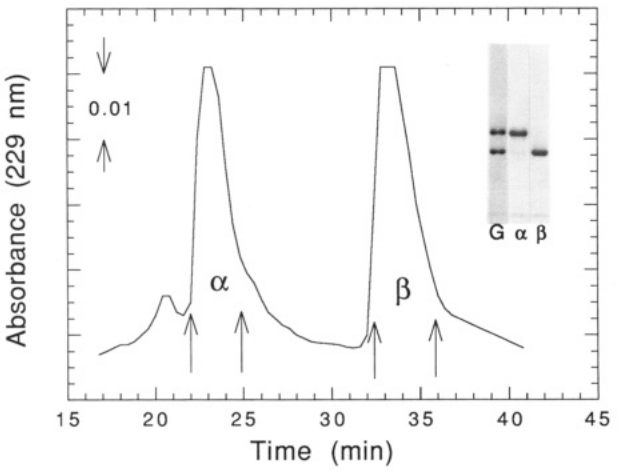

FIGURE 1: Separation of α and β subunits of gizzard tropomyosin (G) by reversed-phase hydrophobic preparative HPLC. Gradient: 41–46% 90% CH_3CN/10% H_2O/1% trifluoroethanol at 0.1%/min. Fractions were pooled between arrows as indicated and identified by SDS–PAGE (see insert).

$A_{277nm} - A_{320nm} = 0.19$ for a 1 mg/mL solution in 0.5 M NaCl, pH 7–8, solutions (Lehrer et al., 1984) and $\Delta A = 0.22$ and 0.17 for $\alpha\alpha$ and $\beta\beta$, respectively, which takes into account that the α chain contains three Tyr and the β chain contains five Tyr (Sanders & Smillie, 1985). Circular dichroism spectra for all three dimers gave values for $[\theta]_{222nm} = 36\,000 \pm 3000$ cm^2 deg $dmol^{-1}$ at 10 °C.

Native GTm was specifically labeled at Cys with pyrene. A 2 mg/mL sample, which had been previously reduced with dithiothreitol, was incubated in 5 M GdmCl/20 mM Hepes buffer, pH 7.5, with pyrenyliodoacetamide (Molecular Probes) in a ratio of 10:1 to protein, added as a small volume in dimethylformamide, for 4 h at room temperature. The unreacted reagent was quenched with excess dithiothreitol. The samples were then treated as described previously (Ishii & Lehrer, 1990) which involved Millipore filtration (0.45 μm) and dialysis, first vs 5 M GdmCl, 20 mM Hepes buffer, pH 7.5, and 0.5 mM dithiothreitol, to remove the unreacted label, and then vs 0.5 M NaCl, 10 mM sodium phosphate buffer, pH 7.0, and 1 mM EDTA, to renature. The degree of labeling was 2.0 mol/mol of pyrene/Tm, determined as described earlier (Ishii & Lehrer, 1990). A sample whose Cys groups were blocked with Nbs_2, and then treated as the unblocked sample, showed no pyrene absorption or fluorescence. A sample which was treated in the absence of GdmCl with label also showed no label incorporation.

Circular dichroism (CD) measurements were performed with an Aviv 60DS spectropolarimeter (Lakewood, NJ) containing a Hewlett-Packard 89100A temperature controller. Ellipticity values at 222 nm vs temperature were obtained automatically in 0.2 °C steps after an equilibration time of 0.3 min and a data averaging time of 10 s, resulting in heating/cooling rates of 0.4 °C/min. To ensure rapid equilibration and accurate temperature measurement, the temperature probe was inserted into the continuously stirred sample solution in a 1-cm cuvette. Fluorescence measurements were performed with a SPEX Fluorolog 2/2/2 photon counting spectrofluorometer (Edison, NJ) in 1-cm cuvette using the thermostated housing. CD and fluorescence data were transferred to a Macintosh computer, analyzed, and plotted with Kaleidograph (Synergy Software, Reading, PA).

Calculations

Consider the two independent dissociation equilibria of the individual homodimers:

$$\alpha\alpha \leftrightarrow 2\alpha$$
$$\beta\beta \leftrightarrow 2\beta \quad (1)$$

The dissociation constants, K_d, are given by

$$K_d = \frac{c^2}{cc} = \frac{c^2}{(cc)_0 - c/2} = 2c_0\left(\frac{\delta^2}{1-\delta}\right) \quad (2)$$

where c and cc are the concentrations of monomers and dimers, respectively, and $c = [\alpha]$ or $[\beta]$. $c_0 = 2(cc)_0$; δ = degree of dissociation $= c/2(cc)_0 = c/c_0$. The subscript 0 denotes the total concentration of the indicated species. From the van't Hoff equation:

$$\ln K_d = \ln 2c_0 + \ln\left(\frac{\delta^2}{1-\delta}\right) = \frac{\Delta G°}{RT} = \frac{\Delta S°}{R} - \frac{\Delta H°}{RT} \quad (3)$$

or

$$\ln\left(\frac{\delta^2}{1-\delta}\right) = \frac{\Delta S°}{R} - \frac{\Delta H°}{RT} - \ln 2c_0 \quad (4)$$

where $\Delta H°$ and $\Delta S°$ are assumed to be independent of temperature over the transition. At the unfolding transition midpoint, $\delta = {}^1/_2$, $T_{1/2}$ is given by

$$\frac{1}{T_{1/2}} = \frac{\Delta S° - R \ln c_0}{\Delta H°} \quad (5)$$

When $c_0 = 1$ M, a standard transition temperature, $T_m°$, can be defined:

$$\left(\frac{1}{T_{1/2}}\right)_{1M} = \frac{1}{T_m°} = \frac{\Delta S°}{\Delta H°} \quad (6)$$

and from eq 5 and 6

$$\frac{1}{T_{1/2}} = \frac{1}{T_m°} - \frac{R \ln c_0}{\Delta H°} \quad (7)$$

It can be seen that the concentration dependence of $1/T_{1/2}$ is greater the smaller the value of $\Delta H°$. Experimentally, it is assumed that δ = the degree of helix unfolding which is determined from CD measurements at 222 nm. The slope of a plot of $R \ln [\delta^2/(1-\delta)]$ vs $1/T$ gives $-\Delta H°$; $T_{1/2}$ is obtained from the plot at $\delta = {}^1/_2$; $T_m°$ is obtained from eq 7 and $\Delta S°$ from eq 4 or 6.

For the dissociation of $\alpha\beta$:

$$\alpha\beta \leftrightarrow \alpha + \beta$$

where

$$K_{\alpha\beta} = [\alpha][\beta]/[\alpha\beta] \quad (8)$$

Because of the possibility of re-formation of homodimers during dissociation of $\alpha\beta$, $\Delta H°(\alpha\beta)$ and $T_m°(\alpha\beta)$ are not calculated from the unfolding profile of $\alpha\beta$ but are obtained by a fitting procedure using a program that takes into account the coupled equilibria of the homodimers (eq 1) and heterodimer (eq 8).

A program was written in FORTRAN F77 which solves for the concentration of each of the species involved in the equilibria of eq 1 and 8 for mixtures of α and β as a function of temperature using the conservation of mass:

$$[\alpha]_0 = [\alpha] + 2[\alpha\alpha] + [\alpha\beta] \quad (9)$$
$$[\beta]_0 = [\beta] + 2[\beta\beta] + [\alpha\beta] \quad (10)$$

Substituting values from eq 2 and 8 into eq 11 and 12 yields

$$2[\alpha]^2/K_{\alpha\alpha} + (1 + [\beta]/K_{\alpha\beta})[\alpha] - [\alpha]_0 = 0 \quad (11)$$
$$2[\beta]^2/K_{\beta\beta} + (1 + [\alpha]/K_{\alpha\beta})[\beta] - [\beta]_0 = 0 \quad (12)$$

Table I: Thermodynamic Quantities Associated with Thermal Unfolding Transitions of the Three Dimers of GTm[a]

dimer[a]	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol)	$T_m°$ (°C)[b]	$T_{1/2}$ (°C)[c]	$\Delta G°$ (kcal/mol)[d]
$\alpha\alpha$	162	490	57.4	40.0	9.0
$\alpha\beta$	225	681	57	43	12.4
$\beta\beta$	98	283	74.0	42.6	9.6

[a] Conditions as for Figure 2. [b] Standard transition temperature (see eq 8). [c] Temperature at 50% helix for 0.5 μM dimer concentration. [d] At 39 °C. [e] Homodimer values from experiment; heterodimer values from best fit to experimental unfolding profile (see text).

where the K's are the dissociation constants for the indicated dimers. These simultaneous quadratic equations are solved by iteration at each temperature for the concentration of monomers after values of $K_{\alpha\alpha}(T)$, $K_{\beta\beta}(T)$, $K_{\alpha\beta}(T)$, and $[\alpha]_0$ and $[\beta]_0$ are specified. Values for the temperature dependence of the dissociation constants in the calculation are obtained from $T_m°$ and $\Delta H°$ measured for $\alpha\alpha$ and $\beta\beta$ dissociation using eq 3 with corresponding $T_m°$ and $\Delta H°$ values estimated for $\alpha\beta$ (see below). A guess for $[\beta]$ is automatically substituted into eq 11 which is then solved for $[\alpha]$ with the quadratic formula. The calculated value for $[\alpha]$ is then substituted into eq 12 to obtain a new value for $[\beta]$. The process is automatically repeated until successive values of both $[\alpha]$ and $[\beta]$ converge to within the desired degree of accuracy. The program calculates the concentrations of dimers with eq 9 and 10, and the temperature dependence of helix content, $1 - \delta = ([\alpha\alpha] + [\beta\beta] + [\alpha\beta])/([\alpha]_0 + [\beta]_0)$, is then fit to the experimental unfolding curve of $\alpha\beta$ by systematically varying values for $T_m°(\alpha\beta)$ and $\Delta H°(\alpha\beta)$ until a close correspondence is obtained. The best-fit values for $T_m°$ and $\Delta H°$ for the heterodimer are listed in Table I along with the values for the homodimers.

Results

Unfolding of $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$. The helix unfolding profiles of the three species of GTm consist of single reversible transitions (Figure 2). These high-resolution CD unfolding curves agree with previous studies of the native $\alpha\beta$ dimer and the $\alpha\alpha$ and $\beta\beta$ dimers (Woods, 1976; Graceffa, 1989; Lehrer et al., 1984; Sanders et al., 1986). The fraction unfolded, δ, was calculated from the ellipticity change at 222 nm corrected for the temperature dependence of the ellipticities of the helical and unfolding forms indicated by the straight lines (Figure 2A). The helix ellipticity had a linear temperature dependence between 10 and 20 °C with a slope of −0.4 %/deg. for all dimers, the same value measured for other α-helices (Hvidt et al., 1985). We assumed negligible temperature dependence of ellipticity for the random-coil in view of the limited data at high temperatures and the small positive slope for the linear dependence of the unfolded helix obtained by Hvidt (+0.05 %/deg on the same scale).

Each species has a different thermal unfolding profile with $\alpha\alpha$ the least stable ($T_{1/2}$ = 40.0 °C). The transition midpoints for $\alpha\beta$ ($T_{1/2}$ = 42.9 °C) and $\beta\beta$ ($T_{1/2}$ = 42.6 °C) were greater than $\alpha\alpha$ and similar to each other, although the slope of the transition for $\beta\beta$ was less than $\alpha\beta$, and $\beta\beta$ was somewhat more stable than $\alpha\beta$ in the high-temperature third of the transition (Figure 3A). From the reversibility of the unfolding/refolding profiles, it is clear that each system was in equilibrium throughout the unfolding/dissociation process. Evidence for the thermal unfolding of $\alpha\beta$ without appreciable formation of homodimers can be seen by noting the lack of agreement of the $\alpha\beta$ unfolding profile with the calculated unfolding profile of a 1:1 mixture of $\alpha\alpha/\beta\beta$ (Figure 3A, solid curve). However,

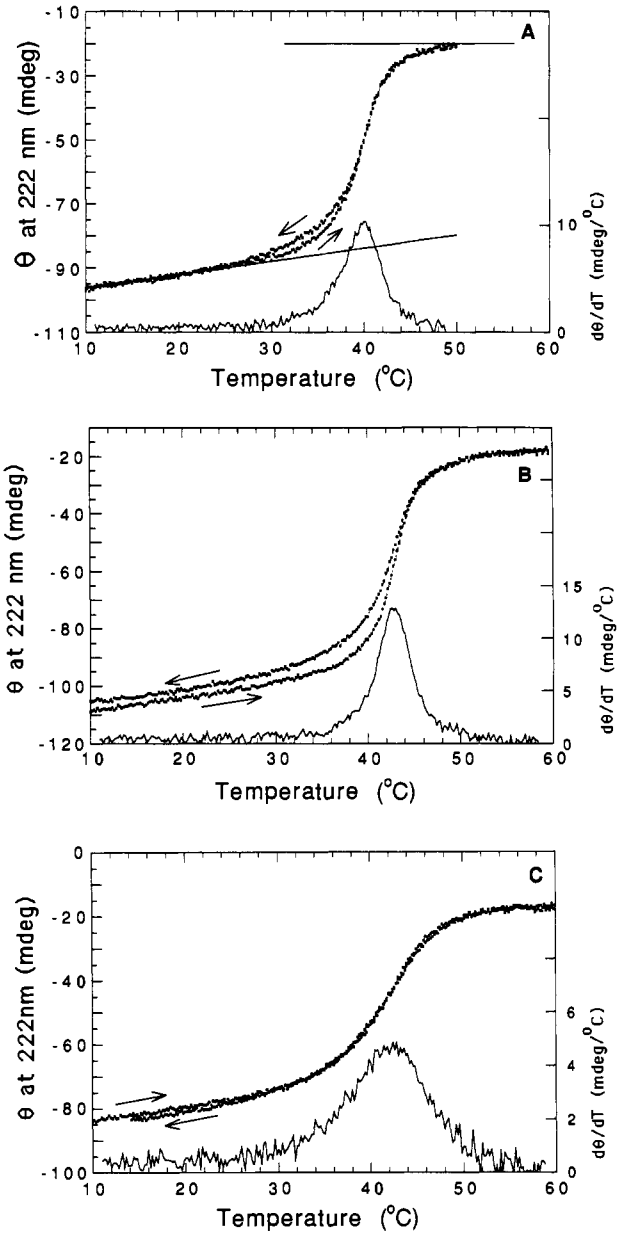

FIGURE 2: Reversible helix thermal transitions of gizzard tropomyosin dimers measures by circular dichroism: ellipticity, θ, and $d\theta/dT$ vs T. (A) $\alpha\alpha$ at 0.50 μM; straight lines indicate initial and final slopes used to calculate the fraction unfolded. (B) $\alpha\beta$ at 0.50 μM (C) $\beta\beta$ at 0.34 μM, in 0.5 M NaCl, 10 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.5 mM dithiothreitol.

a small amount of homodimer refolding does take place, leading to a small difference between the calculated unfolding profile for $\alpha\beta$ and the experimental helix unfolding profile for the whole $\alpha\beta$ system (see below). Plots of $\ln [\delta^2/(1 - \delta)]$ for the homodimers gave straight lines (Figure 3B) from which it was possible to calculate values for $\Delta H°$, $\Delta S°$, and $T_m°$ (see Calculations and Table I).

It has been shown that the stability of tropomyosin is appreciably decreased in low-salt solutions (Betteridge & Lehrer, 1983) and differences between the stability of the dimers is made more apparent (Figure 4). Thus, $\alpha\alpha$ is the least stable of the three species with $T_{1/2}$ = 32 °C, but, in contrast to high-salt solutions, $\beta\beta$ is somewhat less stable than $\alpha\beta$ ($T_{1/2}$ = 36 and 38 °C, respectively). The stability of a given dimer decreases with increases in pH at low salt (Qian & Lehrer, 1990) without changing the order of stability, suggesting that it is the net charge that is important in determining stability.

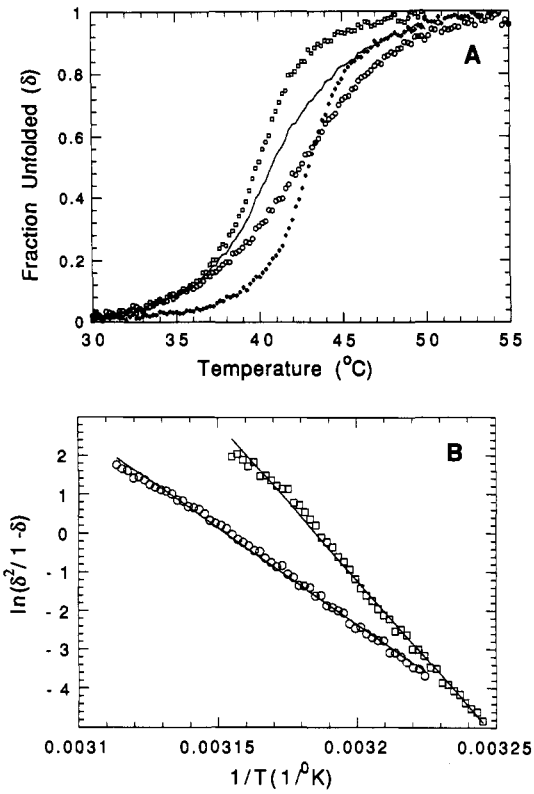

FIGURE 3: (A) Unfolding profiles of gizzard tropomyosin dimers from normalized corrected data of Figure 2: $\alpha\alpha$ (□); $\beta\beta$ (○); $\alpha\beta$ (♦); $\alpha\alpha + \beta\beta$ calculated (—). (B) van't Hoff plots of $\alpha\alpha$ (□) and $\beta\beta$ (○) unfolding/dissociation transitions with least-squares straight line fits.

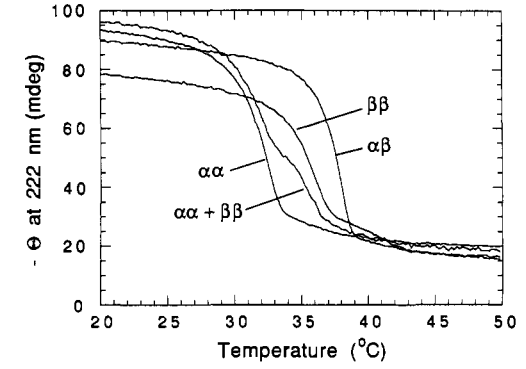

FIGURE 4: Helix thermal transitions of gizzard tropomyosin dimers and a 50:50 mixture of $\alpha\alpha/\beta\beta$ at low ionic strength. $[\alpha\alpha]$ = 0.45 μM, $[\beta\beta]$ = 0.39 μM, $[\alpha\beta]$ = 0.45 μM, $[\alpha\alpha]$ and $[\beta\beta]$ in mixture, 0.23 and 0.23 μM, respectively, in 2 mM sodium phosphate buffer, pH 7.00 ± 0.05, and 0.5 mM dithiothreitol.

The order of stability of the dimers in low salt at a given pH is in agreement with the number of optimum ionic interactions of charged residues across the chains (Sanders & Smillie, 1985). A 1:1 mixture of the two homodimers shows two equal transitions corresponding to unfolding of the separate species, in agreement with earlier work (Graceffa, 1989; Lehrer & Qian, 1990) indicating lack of subunit exchange to form $\alpha\beta$ during unfolding at low ionic strength conditions. This is probably due to a slower exchange rate at the lower ionic strength due to less efficient shielding of the net repulsive charge on the molecule. Most of the original ellipticity was recovered on cooling, but the refolding curves lagged in temperature presumably due to the slower reassociation rates at these low ionic strengths. It should be pointed out that at these low protein concentrations, little or no low salt-induced polymerization is expected (Asai, 1961).

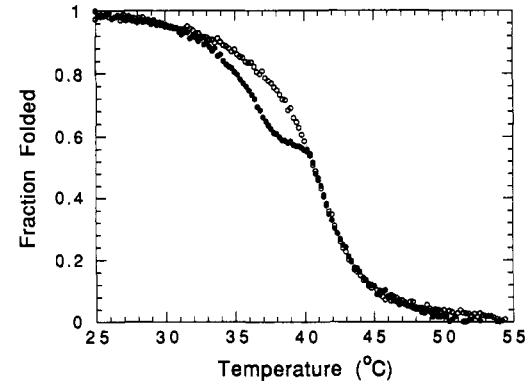

FIGURE 5: Helix unfolding profile of a 50:50 mixture of homodimers near physiological salt conditions: heating curve (●); reheating curve (○); 0.5 μM in 0.05 M sodium acetate, 10 mM sodium phosphate buffer, 1 mM EDTA, and 0.5 mM dithiothreitol.

In contrast to low-salt conditions, at about 0.05 M and higher salt concentrations, a 1:1 mixture of $\alpha\alpha$ and $\beta\beta$ undergoes chain exchange to form $\alpha\beta$ during the unfolding process at temperatures where appreciable amounts of $\alpha\alpha$ unfold and dissociate. This kinetic process results in a truncated $\alpha\alpha$ transition (Graceffa, 1989; Lehrer & Qian, 1990). The temperature at which the $\alpha\alpha$ unfolding curve abruptly changes slope depends upon the rate of heating compared to the rate of chain exchange (Lehrer & Qian, 1990). At 0.05 M sodium acetate concentration (Figure 5), the $\alpha\alpha$ unfolding transition proceeds to somewhat greater temperatures than at 0.5 M salt before appreciable exchange takes place, in agreement with the decreased electrostatic shielding at this lower ionic strength which may be expected to lower the rate of reassociation of the dissociated subunits.

Unfolding of Pyrene-Labeled Dimers. Further evidence for chain exchange to form $\alpha\beta$ from mixtures of $\alpha\alpha + \beta\beta$ was obtained with fluorescence measurements on pyrenyliodoacetamide-labeled GTm. It was previously shown that refolding of GdmCl-denatured native Tm at low temperature results in the formation of a 1:1 mixture of homodimers (Graceffa, 1989). Because native GTm was labeled with pyrene in GdmCl, a 1:1 mixture of pyrene–$\alpha\alpha$ and pyrene–$\beta\beta$ is expected on renaturation. This should result in excimer fluorescence because each chain contains one labeled Cys residue in equivalent positions (Cys-190's for $\alpha\alpha$ and Cys-36's for $\beta\beta$) but are too far apart in the heterodimer (Cys-36 and Cys-190) to result in excimer fluorescence. As expected for homodimers, excimer fluorescence was observed at 25 °C. It decreased markedly on heating above about 38 °C over time and did not recover after cooling to 25 °C (Figure 6A), despite the recovery of the helical content (Figure 6B). Thus, under these high-salt conditions at physiological temperature, chain exchange took place to form pyrene-labeled $\alpha\beta$ as occurs for unlabeled $\alpha\beta$. It should be pointed out that the labeling procedure did not significantly affect the general unfolding properties of GTm. The CD unfolding profile of the labeled homodimers showed a truncated $\alpha\alpha$ transition as for unlabeled homodimers and a reversible unfolding/refolding profile for the labeled $\alpha\beta$ that was formed by chain exchange (Figure 6B). The $T_{1/2}$ for the pyrene–$\alpha\beta$ so formed was 2 °C lower than unlabeled $\alpha\beta$, indicating some decrease in stability produced by the label under these conditions. The pyrene–$\alpha\beta$ formed by chain exchange showed a monotonic decrease of monomer fluorescence with temperature with a slight indication of a fluorescence transition between 45 and 50 °C in the main helix unfolding transition. There was no increase in excimer fluorescence, indicating a lack of significant for-

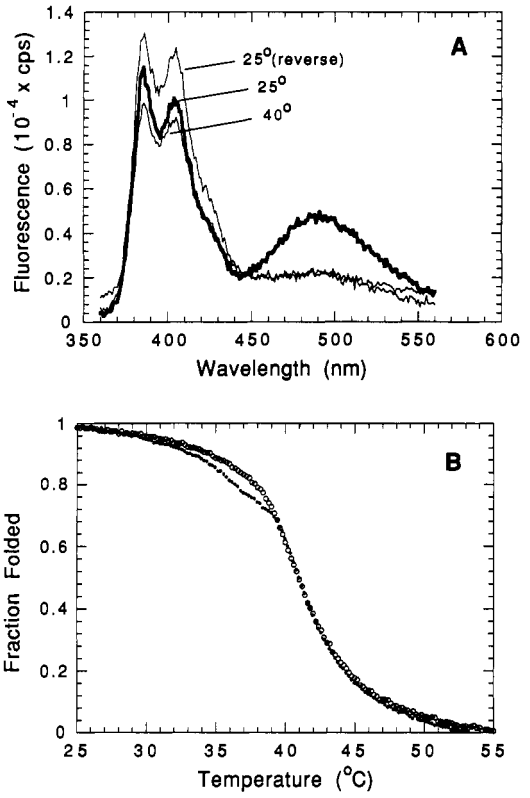

FIGURE 6: Fluorescence and CD properties of a 50:50 mixture of pyrene-labeled homodimers, 0.25 μM αα + 0.25 μM ββ. (A) Fluorescence spectra showing excimer fluorescence (490-nm band) in the homodimer mixture and irreversible loss of excimer fluorescence at 40 °C due to subunit exchange; λ_{exc} = 340 nm. (B) Helix unfolding profiles of the homodimer mixture showing irreversible subunit exchange: heating curve (●); reheating curve (○). Conditions as for Figure 2.

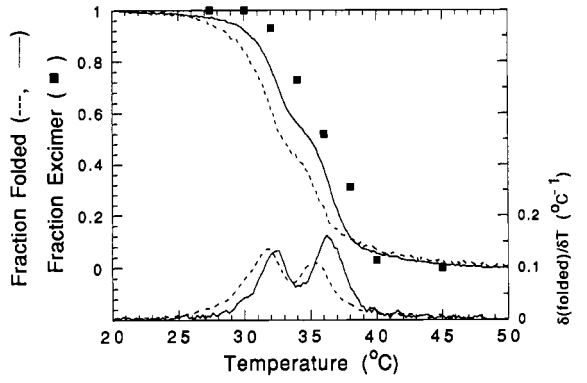

FIGURE 7: Effect of pyrene label on thermal unfolding profiles of αα + ββ at low salt. Unlabeled αα + ββ unfolding (---); pyrene αα + ββ unfolding (—); excimer fluorescence (■). Conditions as for Figure 4.

mation of homodimers during the αβ unfolding transition.

In low-salt solution where the 1:1 mixture of unlabeled αα and ββ unfolded independently without chain exchange (Figure 4), a mixture of pyrene–αα/pyrene–ββ also showed independent unfolding, but the profile was shifted about 1 °C to higher temperature compared to unlabeled dimers (Figure 7). This indicated that in contrast to pyrene–αβ, where the pyrenes caused a slight destabilization, for pyrene–αα and pyrene–ββ the attached pyrenes slightly stabilized the molecules. These effects are apparently due to hydrophobic interaction between the pyrenes in the homodimers. The excimer fluorescence of the mixture of labeled homodimers was also lost in two apparent equal transitions, as expected, but interestingly, the

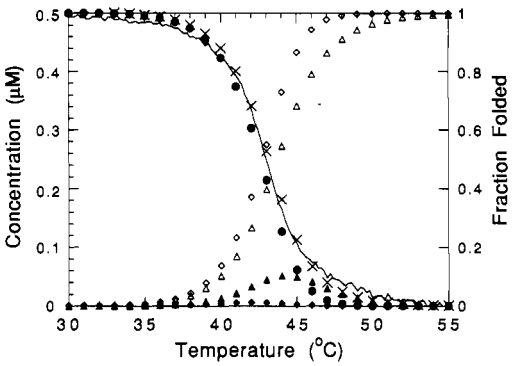

FIGURE 8: Temperature dependence of the species present at equilibrium calculated from thermodynamic parameters obtained from the unfolding profiles of the dimers for $[\alpha]_0 = [\beta]_0 = 0.5$ μM. αβ (●), αα (♦), ββ (▲), α (◇), β (△), fraction folded (×). Experimental unfolding profile of αβ (—).

profile was shifted about 1.5 °C to higher temperature compared to the helix transition, resulting in temperatures where there is a persistence of excimer fluorescence when the helix was fully lost (Figure 7). This suggests that the pyrene–pyrene interaction is able to maintain chain association to higher temperatures even though the chains are unfolded. As shown above, at higher ionic strengths, chain exchange results in a time-dependent loss of excimer fluorescence. Kinetic studies showed that the half-time for the exchange (pyrene–αα + pyrene–ββ → 2 pyrene–αβ) at 38 °C and 0.5 M salt concentration was about 6× longer when measured by the time-dependent loss of excimer fluorescence than when measured by the increase of ellipticity at 222 nm. Thus, effects of the pyrene–pyrene interaction which are present in the homodimers but absent in the heterodimer seemed to explain the lag of excimer loss relative to the helix loss.

Calculation of αβ Unfolding. To determine if thermodynamics can explain the preferential formation of heterodimers, we calculated the temperature dependence of the equilibrium concentration of all of the species present using $\Delta H°$ and $T_m°$ values for the unfolding of the three dimeric species of GTm (αα, αβ, and ββ) and the degree of helix loss with temperature. This calculation assumes that dissociation into subunits occurs in parallel with unfolding and that $\Delta H°$ is constant over the transition (see Calculations). Using experimental values obtained for $\Delta H°$ and $T_m°$ for αα and ββ, we obtained corresponding values for αβ that gave best agreement with the experimental helix unfolding profile, with $\alpha_0 = \beta_0 = 0.5$ μM, similar to concentrations used in the above experiments (Figure 8). Values of $\Delta H°$ and $T_m°$ for the heterodimer could be estimated quite accurately because the midpoint and the slope of the calculated unfolding curve were quite sensitive to $T_m°$ and $\Delta H°$, respectively, allowing a good fit of the calculated to the experimental curve. The calculations show that below 40 °C, where the system is completely folded, the calculated concentrations of αα and ββ are effectively nil, leading to the prediction that αβ is the preferentially assembled equilibrium species. Over the entire transition region, the calculated unfolding of helix corresponds closely to the experimental unfolding of αβ. There is a small indication of some re-formation of ββ in the second half of the main transition, in agreement with the greater stability of ββ in that region.

From the thermodynamic constants measured, the dissociation free energy for each species at 39 °C (fowl body temperature) was calculated (Table I). $\Delta G°_e$ for the exchange equilibrium, αα + ββ = 2αβ, should be much less than zero in order to complete homodimer exchange to heterodimers to take place. Expressing $\Delta G°_e$ in terms of the individual ΔG's

for the dissociation reactions:

$$\Delta G^\circ_e = \Delta G^\circ_{\alpha\alpha} + \Delta G^\circ_{\beta\beta} - 2\Delta G^\circ_{\alpha\beta}$$

For $\Delta G^\circ_e \ll 0$: $\Delta G^\circ_{\alpha\beta} - [1/2(\Delta G^\circ_{\alpha\alpha} + \Delta G^\circ_{\beta\beta})] = 5.8$ kcal/mol $\gg 0$ (Table I).

Discussion

Previous studies have shown that the native GTm molecule is composed of two subunits, α and β, which differ slightly in amino acid sequence (Lau et al., 1985; Sanders & Smillie, 1985), present as $\alpha\beta$ heterodimers (Sanders et al., 1986). It appears that the subunits can refold by two different pathways leading to a different dimer composition depending upon the refolding conditions. Refolding an equimolar mixture of $\alpha + \beta$ subunits from denaturants during low-temperature dialysis produces a 1:1 mixture of homodimers (Graceffa, 1989), in contrast to slowly cooling from 60 °C under physiological salt concentration and greater, which only produces $\alpha\beta$ (Figure 2B, and 8). Refolding by rapid cooling from high temperature also favors homodimers (Lehrer & Qian, 1990). Our previous studies showed that the heterodimer can slowly form from a homodimer mixture by subunit exchange, when incubated at physiological temperature, but the rate of subunit exchange is very slow in low-salt solutions probably as a result of lack of sufficient shielding of the net subunit charge (Lehrer & Qian, 1990). These observations have been extended here with equilibrium CD and fluorescence studies on unlabeled and pyrene-labeled GTm dimers.

The concept that the heterodimer is preferentially formed at equilibrium from mixtures of α and β subunits was verified in this study with calculations using unfolding/dissociation constants obtained from the temperature dependence of the cooperative transitions of the purified homodimers, $\alpha\alpha$ and $\beta\beta$. The calculations assume that the transitions are single, reversible, and two-state and that ΔH° is temperature-independent (see above equations). The CD data were reversible over the entire transition in each case, and van't Hoff plots were linear with no indication of appreciable intermediate formation, indicating that the approximations are reasonable over the limited temperature range of the transition. It was also assumed that dissociation into subunits occurs in parallel with unfolding. Previous CD and sedimentation studies with GdmCl unfolding (Pont & Woods, 1971) and CD studies of temperature-induced unfolding of striated muscle tropomyosin at different concentrations have provided evidence for dissociation in the main unfolding transition (Holtzer et al., 1983; Stafford, 1985). Fluorescence and light-scattering data also supported dissociation (Graceffa & Lehrer, 1980; Yukioka et al., 1985). The above experiments with pyrene–GTm at low salt which show the correlation of the excimer loss profile with the unfolding profile provide further evidence for the parallel course of dissociation with unfolding.

Equation 7 gives an explicit concentration dependence of $T_{1/2}$ which depends on ΔH° (see Calculations). In one experiment with $[\alpha\beta]_0$ at 34 μM, we did observe a 2 °C increase in the value of $T_{1/2}$ compared to the 0.5 μM value, somewhat lower than the 4 °C calculated with eq 7. The smaller observed difference may be due to the temperature dependence of ΔH° which was neglected in the calculations. A clear example of the concentration dependence of stability has recently been reported for another coiled-coil system. The 33-residue coiled-coil "leucine-zipper" homodimer peptides, GCN4-P1 and GCN4-P1N, show a concentration dependence of $T_{1/2}$ (O'Shea et al., 1989, their Figure 3). When their data and eq 7 were used, linear plots of $1/T_{1/2}$ vs $R \ln c_0$ were obtained, in agreement with the above theory. The plots gave values of $\Delta H^\circ = 50$ kcal/mol for both peptides and $T_m^\circ = 104$ and 108 °C for GCN4-P1 and GCN4-P1N, respectively. The greater concentration dependence on $T_{1/2}$ for this system compared to tropomyosin is due to the smaller ΔH°.

Within the above reasonable approximations, it is seen that the calculations predict that the heterodimer is the preferred equilibrium species at physiological temperature and below, and unfolds/dissociates without appreciable formation of homodimer species. The values in Table I indicate that $\alpha\alpha$ is less stable than $\beta\beta$ mainly due to a greater ΔS° of unfolding. The greater stability of $\alpha\beta$ compared to the mixture of homodimers, however, appears mainly to be the result of a greater ΔH° of unfolding. These differences in dimer stability will ultimately be explained by the different specific amino acid side chain interactions across the chains.

The predicted greater concentration dependence of $1/T_{1/2}$ for $\beta\beta$ compared to the other dimers due to its small value of dissociation, ΔH° (eq 7), may provide an explanation for the observed formation of homodimers after renaturation from GdmCl solutions at the high concentrations of tropomyosin used, i.e., 1–5 mg/mL (Graceffa, 1989). At 15 μM (1 mg/mL), the $T_{1/2}$ for $\beta\beta$ is calculated to be 4 °C greater than that $T_{1/2}$ for $\alpha\beta$ compared to their near-equal value at 0.5 μM. If $\beta\beta$ is relatively more stable than $\alpha\beta$ at higher protein concentrations with denaturant-induced unfolding as predicted for thermal unfolding, it would be expected that $\beta\beta$ would preferentially form over $\alpha\beta$ as the denaturant is dialyzed out. At the low temperature of dialysis ($\approx$5 °C), if subunit exchange does not readily occur, $\alpha\alpha$ would form after $\beta\beta$ as the concentration of denaturant further decreases. This would result in a nonequilibrium mixture of homodimers which could then convert to the equilibrium heterodimer at greater temperature, as observed.

In general, the equilibrium concentration distribution of coiled-coil dimers, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, for an equal mixture of subunits will depend upon the dissociation constants or relative stability under a given set of conditions, e.g., pH, ionic strength, temperature, total subunit concentration, etc.. Recent studies with frog skeletal tropomyosin have also shown that the heterodimer is preferred in vitro, at equilibrium at temperatures within the physiological range (Lehrer et al., 1989). In contrast, previous refolding studies with subunits of rabbit skeletal tropomyosin have yielded a random composition (Holtzer et al., 1984) or a preference toward the heterodimer (Brown & Schachat, 1985). If equilibrium was reached, these differing results may reflect the sensitivity of similar dimer dissociation constants to the precise conditions of the experiment.

In summary, our studies offer a thermodynamic approach which may explain why many coiled-coil molecules and molecules with coiled-coil domains assemble preferentially as heterodimers (Bronson & Schachat, 1982; Coulombe & Fuchs, 1990; Hatzfeld & Weber, 1990; Steinert, 1990; Lehrer et al., 1989; O'Shea et al., 1989; Sanders et al., 1986). These studies also verify that the thermodynamically preferred heterodimer can form via subunit exchange after assembly as homodimers. Thus, if homodimers are the first dimers to be assembled in a cell, heterodimers can later be formed via chain exchange at equilibrium under conditions where the relative stability of the heterodimer is greater than the average stability of the homodimers. On the basis of calculations, a suggestion is also offered for the observed formation of homodimers of tropomyosin from frog and gizzard at high protein concentrations when refolding takes place at low temperature. Ongoing

studies indicate that a similar thermodynamic explanation for heterodimer assembly also applies in the case of tropomyosin from *Rana temporaria* (Lehrer, Qian, and Hvidt, unpublished experiments).

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The Elasticity of Synthetic Phospholipid Vesicles Obtained by Photon Correlation Spectroscopy

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ABSTRACT: Osmotic-swelling experiments were conducted on a variety of preparations of "uniform" unilamellar vesicle systems. The synthetic lipid preparations included both vesicles produced by extrusion through polycarbonate ultrafiltration membranes and vesicles produced by the pH-adjustment method. The vesicles were monitored by photon correlation spectroscopy during swelling as the osmolarity of the external solution was decreased. Contrary to our previously reported results [Aurora, T. S., Li, W., Cummins, H. Z., & Haines, T. H. (1985) *Biochim. Biophys. Acta 820*, 250-258; Li, W., & Haines, T. H. (1986) *Biochemistry 25*, 7477-7483; Li, W., Aurora, T. S., Haines, T. H., & Cummins, H. Z. (1986) *Biochemistry 25*, 8220-8229; Haines, T. H., Li, W., Green, M., & Cummins, H. Z. (1987) *Biochemistry 26*, 5439-5447] large unilamellar vesicles produced from acidic lipids by the pH-adjustment technique were highly polydisperse and did not swell in a manner that permitted the computation of a Young's modulus, presumably due to the polydispersity. Also contrary to our previous reports, membranes derived from bovine submitochondrial particles did not produce evidence of swelling when subjected to similar protocols. Analysis of osmotic swelling of extruded unilamellar vesicles has allowed us to assign Young's moduli for bilayers of dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol, in the range (5-8) $\times 10^8$ and (3-6) $\times 10^8$ dyn/cm^2, respectively. The diameters and polydispersites obtained with electron microscopy and photon correlation spectroscopy were compared directly and with computer-modeling techniques. While excellent agreement was obtained for distributions with low polydispersity ($\lesssim$0.1), serious disagreement was found when the polydispersity exceeded $\sim$0.2.

Osmotic properties of unilamellar phospholipid vesicles have become increasingly important as our understanding of transmembrane proteins emerges. The exploration of mechanicoselective ion channels in the last five years as the patch-clamp technique was applied to biological membranes has shown that these channels are present in virtually all of the cells investigated (Morris, 1990). The data suggest that these channels

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