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Unfolding/Refolding Studies of Smooth Muscle Tropomyosin
EVIDENCE FOR A CHAIN EXCHANGE MECHANISM IN THE PREFERENTIAL ASSEMBLY OF THE NATIVE HETERODIMER

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Sherwin S. Lehrer‡‡ and Yude Qian¶
From the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114 and the Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

The thermal and the urea-induced unfolding profiles of the coiled-coil α-helix of native and refolded tropomyosin from chicken gizzard were studied by circular dichroism. Refolding of tropomyosin at low temperature from α + β subunits, dissociated by guanidinium chloride, urea, or high temperature, predominantly produced αα + ββ homodimers in agreement with earlier studies of refolding from guanidinium chloride (Graceffa, P. (1989) Biochemistry 28, 1282-1287). The presence of two unfolding transitions in low salt solutions with about equal helix loss verified the composition with the first unfolding transition of the homodimer mixture originating from αα. In contrast, refolding by equilibrating at temperatures close to physiological, however, produced the native αβ heterodimer, which unfolded in a single transition. The refolding kinetics of dissociated α + β subunits indicated that ββ homodimers form first, leading to αα homodimers both of which are relatively stable against chain exchange below ~25°C. Equilibrating the homodimer mixture at 37-40°C for long times, however, produced the native αβ heterodimer, which unfolded in a single transition. The equilibria involved indicate that the free energy of formation from subunits of αβ is much less than that of (αα + ββ)/2. In vivo folding of αβ from the two separate α and β gene products is, therefore, thermodynamically favored over the formation of homodimers and biological factors need not be considered to explain the native preferred αβ composition.

Smooth muscle tropomyosin, purified from chicken gizzard (GTm), is composed of two subunits, α and β, which differ slightly in amino acid sequence (1, 2) and are present in about equal amounts (3). Because tropomyosin (Tm) is a two-subunit molecule, assembly in vivo after chain biosynthesis or in vitro after chain dissociation, for α/β = 1 can, in principle, either produce a 1:1 mixture of homodimers (αα/ββ), all heterodimer (αβ), or a mixture of the three species. Evidence has been presented that GTm isolated under native conditions is a heterodimer (4) but that after refolding by dialysis at low temperature from GdmCl-dissociated chains, a 1:1 mixture of homodimers is produced (5). This apparent discrepancy between the native and in vitro refolded composition raises the possibility that the in vivo assembly of the molecule may require biological factors rather than being determined by thermodynamics alone.

Tm is a coiled-coil α-helix that dissociates into subunits in parallel with a major cooperative helix-coil transition (6, 7), whose transition midpoints are characteristic of the species and muscle type as well as the molecular composition (8). Thus, information about Tm composition and subunit dissociation/association may be obtained by monitoring the characteristic unfolding profiles of the homodimers and heterodimer using circular dichroism (CD) techniques. Previous CD studies of the unfolding of GTm showed a single transition for the native molecule (4, 8, 9).

In this work we confirm that assembly of GTm at low temperature from unfolded α and β subunits in denaturants produces αα + ββ (5) and show that rapid refolding at low temperature from the separated chains at high temperature also predominantly produces homodimers. In contrast, refolding by incubating at temperatures close to physiological produces the native αβ heterodimer. Thus, αβ is the preferred species in vitro, and biological factors are not required to explain its preference in vivo. CD studies also provided information about the mechanism of assembly by showing: (i) that homodimers are initially formed from dissociated subunits in a rapid process; (ii) that αβ is produced from the mixture of homodimers between 37-40°C via a chain exchange mechanism, during times that allow equilibrium to be reached.

MATERIALS AND METHODS

Native smooth muscle GTm was prepared from acetone powder of chicken gizzard tissue at temperatures below 5°C and characterized as described earlier (9). Refolded GTm was prepared from solutions of ~3 mg/ml native GTm by incubating with 5-6 M GdmCl (Schwartz-Mann, ultra pure, added as solid), 5-10 mM dithiothreitol, 1 mM EDTA, 10 mM sodium phosphate buffer, pH 7.0 for 1 h at room temperature and dialyzing at 5°C versus 0.5 mM NaCl, 2 mM sodium phosphate, pH 7.0, 1 mM EDTA to assemble. Enriched ααGTm was prepared from refolded GTm by hydroxyapatite chromatography (10), using a 1 × 8-cm column to which 5 mg of protein was applied in starting buffer (0.1 M sodium phosphate, pH 7.2, 0.5 mM NaCl, 1 mM EDTA) and eluted with a linear gradient consisting of 50 ml of starting buffer and 60 ml of ending buffer (0.35 M sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA).

* The nomenclature for the smooth Tm subunits differs among different groups: α, β, this manuscript, Ruiz-Opazo and Nadal-Ginard (1987) J. Biol. Chem. 262, 4755-4765; γ, β, Sanders and Smillie (2); γ, a, (Helfman et al. 1984) J. Biol. Chem. 259, 14136-14143.

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3 To whom reprint requests should be addressed: Dept. of Muscle Research, Boston Biomedical Research Inst., 20 Stanford St., Boston, MA 02114.

4 Current address: Chemistry Dept., Hangzhou University, Peoples Republic of China.

5 The abbreviations used are: GTm, smooth muscle tropomyosin; purified from chicken gizzard; Tm, tropomyosin; GdmCl, guanidinium chloride; Nbs, 5,5'-dithiobis-2-nitrobenzoate; SDS, sodium dodecyl sulfate.
pH 7.2, 0.5 M NaCl, 1 mM EDTA). One broad peak was obtained with the αα in the trailing limb which was pooled and dialyzed for use. Low salt stock solutions (1-3 mg/ml) of native and refolded and enriched GTm could be stored on ice for several weeks after dialysis versus 2 mM sodium phosphate, pH 6.0-7.0. 1 mM EDTA or frozen for months without changes in properties.

Disulfide crosslinking with 5,5'-dithiobis-2-nitrobenzoate (Nbs), 2,2'-dithiopyridine, and 4,4'-dithiopyridine (Aldrich) were performed as outlined previously (11).

CD measurements were performed with an Aviv 60DS spectropolarimeter (Lakewood, NJ) containing a Hewlett-Packard 89100A temperature controller. Thermal data were obtained automatically as ellipticity values at 222 nm in 0.2-nm steps with an equilibration time of 0.3 min and a data averaging time of 10 s at each temperature step with 1-cm cuvettes using the internal magnetic stirrer and the temperature probe inserted into the solution to insure uniformity and rapid temperature equilibration during temperature changes.

SDS-polyacrylamide gel electrophoresis was run in a Bio-Rad minigel apparatus at 9% acrylamide with a Laemmli-stacking buffer system in the absence of reducing agent.

RESULTS

In these studies, three samples of GTm were used: GTm (αβ),2 GTm refolded from 6 M GdmCl (αα + ββ), and an enriched αα sample (80% αα, 20% ββ). SDS-polyacrylamide gel electrophoresis of the samples before and after crosslinking with aromatic disulfides at low concentration (to optimize cross-linking over the competitive blocking reaction) (12) verified their composition (Fig. 1). It is seen that native GTm reacts with the disulfide reagents but is not cross-linked. This is because the α-chain contains a Cys at position 190 and the β-chain contains a Cys at position 36 (1, 2) and in the αβ molecule where the chains are parallel and in register, the 2 Cys are too far apart to be disulfide cross-linked. In contrast, the refolded αβ GTm is appreciably cross-linked and is composed of an equal mixture of αα and ββ (5).

The CD spectrum of native gizzard Tm is typical of an α-helix with negative ellipticity peaks at 222, 208, and a positive peak at 193 nm. The value of [θ]222 nm =3.6 × 104 deg cm2/dmol at 15 °C for the mean residue ellipticity indicates >95% α-helix. In low salt solutions, the urea-induced unfolding of native GTm took place in a single cooperative transition with a midpoint at 0.85 M urea; refolded GTm unfolded in two approximately equal transitions at 0.4 and 0.85 M urea (Fig. 2A). The first transition of refolded GTm is identified as being due to the unfolding of αα, since the major transition of enriched αα occurred at ~0.4 M urea. The second transition of refolded GTm must therefore be the unfolding of ββ. Native GTm (αβ) unfolds in a single transition with a midpoint similar to that of the second transition of refolded GTm (due to ββ) indicating that αβ has a stability similar to that of ββ in agreement with thermal unfolding studies at high salt (see below and Ref. 4). These CD data, which show that αα can be distinguished from αβ and ββ by its unfolding profiles in low salt solutions, verifies that refolding from denaturants at low temperature predominantly produces homodimers. Similar CD results were obtained on these samples in studies of thermal unfolding in low salt solutions (Fig. 2B). By comparison with the unfolding of native rabbit skeletal Tm which is

![Fig. 1. SDS-polyacrylamide gel electrophoresis of native and refolded GTm reacted with aromatic disulfides. Unreacted samples (left two lanes): (αα) = enriched by chromatography of refolded GTm; NTm = native GTm. Reacted samples (right six lanes): native GTm and refolded GTm from GdmCl at 5 °C were reacted with Nbs a, 2,2'-dithiopyridine (b), 4,4'-dithiopyridine (c). Reaction conditions: 0.35 mg/ml GTm in 0.5 M NaCl, 2.0 mM sodium phosphate, pH 7.0, 1 mM EDTA, reacted with 0.5 mM disulfide for 5 h at 25 °C and 20 mM iodoacetamide was added prior to run. 5-μg samples applied.](https://www.jbc.org/)

![Fig. 2. Relationship between the helix-unfolding profiles of Tm in low salt solutions and its subunit composition. A, urea-unfolding profiles, 25 °C. B, thermal-unfolding profiles. NG(αβ) = native GTm, RG(αα + ββ) = renatured from GdmCl at 5 °C, RG(0.8αα + 0.2ββ) = enriched αα, NS = native rabbit skeletal Tm(0.6αα + 0.4ββ). 0.05 mg/ml in 2.0 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 25 °C.](https://www.jbc.org/)
known to be a mixture of $\alpha\alpha$ and $\alpha\beta$ (10, 12), these data show that the stability of the various species increases in the order: $\alpha\alpha$ gizzard Tm $< \alpha\beta$ skeletal Tm $\approx \alpha\beta$ gizzard Tm, $\beta\beta$ gizzard Tm $< \alpha\alpha$ skeletal Tm. From these data alone it is difficult to determine the order of stability of $\alpha\beta$ GTm and $\beta\beta$ GTm. This order of stability at low salt is in approximate agreement with the number of optimum ionic interactions of charged residues across the chains (2).

In contrast to refolding at low temperature where homodimers are preferentially produced, when native GTm was refolded at temperatures in the physiological region, $\alpha\beta$ was preferentially produced. Thus, the thermal unfolding profile in low salt solutions at 35-38 °C had been refolded by dialysis at 35-38 °C showed only one transition similar to native GTm (Fig. 3, R 35-38 °C). In a parallel experiment, refolding by dialysis at 5 °C produced an appreciable amount of homodimers as evidenced by two transitions. Rapidly cooling (<2 min) of separated $\alpha + \beta$ chains from 50 to 20 °C in low salt solutions, also resulted in considerable homodimer formation (Fig. 3, R' 20 °C). Similar low salt unfolding studies performed on samples refolded by dilution from urea showed preferential formation of homodimers at 0 °C and heterodimer at 37 °C. These data indicate that refolding at physiological temperature results in the formation of $\alpha\beta$ in contrast to refolding at lower temperatures which favors homodimers.

Further insight into the factors that determine the composition of the dimer was obtained by thermal unfolding/refolding studies of solutions containing salt concentrations close to physiological. The thermal unfolding profile of refolded GTm ($\alpha\alpha + \beta\beta$) between ionic strengths of 0.05 and 1.0 showed two transitions as observed at low salt concentrations (Fig. 2), but the first transition was less prominent and appeared truncated (Fig. 4A, curve 1) as compared with the corresponding low salt transition, in agreement with the data of Graceffa (5). The first part of the first transition coincided with the unfolding of enriched $\alpha\alpha$ by heating up to about 39 °C, but after about 15-20% helix loss, the rest of the curve was very similar to the profile of native $\alpha\beta$ GTm (Fig. 4, curve 2). In view of the above observation that $\alpha\beta$ is preferentially formed during equilibration in the 35-40 °C temperature range in physiological buffer, it appears that the truncated first transition is due to two processes, $\alpha\alpha = 2\alpha\alpha$ and $\alpha\beta = 2\alpha\beta$, i.e. chain exchange which converts homodimers, $\alpha\alpha + \beta\beta = 2\alpha\beta$. This can explain the smaller decrease in helix content observed in the first transition (the truncation), since partially unfolded $\alpha\alpha$ is converted to fully folded $\alpha\beta$ in this temperature range. The second transition must then be due to $\alpha\beta = \alpha + \beta$. The shape of the first transition would then depend on the relative rates of chain exchange versus heating, e.g. a faster heating rate would favor more $\alpha\alpha$ unfolding before chain exchange. To obtain information on the rates involved in refolding and chain exchange, the temperature was quickly dropped to 37 °C from a higher temperature where the chains were separated, and the temperature and ellipticity were monitored with time. It was found that the ellipticity changed in two steps; an initial rapid decrease in parallel with the temperature drop and a very slow exponential decrease (increase in helix content) with a $t_1/2$ of about 500 s after the temperature reached 37 °C (in about 60 s) (data not shown). After incubating at 37 °C for about 1200 s,
the sample was cooled to 20 °C, to quench the exchange process. Its unfolding profile indicated that it was converted to αβ (Fig. 4A, curve 2). More definitive evidence for refolding to αβ in two steps was obtained by a repeat of the kinetic refolding studies at 40 °C, where the difference between the unfolding curves of homodimers and heterodimer is greatest (in Fig. 4A, compare curves 1 and 2). It is clearly seen that the negative ellipticity increased in two processes; a fast process which follows the temperature drop and a slow process with τs ≈600 s (Fig. 4B). It appears that the fast process is the production of homodimers in view of the smaller value of negative ellipticity initially obtained since αα is ≈50% unfolded at 40 °C. The slow process is chain exchange resulting in the production of αβ. Evidence for the fast process producing homodimers was also obtained by monitoring the unfolding profile of a sample which was cooled from 55 to 20 °C rapidly (0.3 °C/s). Although there was incomplete refolding during this fast temperature drop, it can be seen that an appreciable amount of homodimer was formed as evidenced by the presence of the truncated first transition (Fig. 4A, curve 3).

**DISCUSSION**

These data indicate that assembly of α + β subunits by rapid cooling forms homodimers. This is most simply explained by formation of β first then αα since ββ appears to be most stable. In the physiological temperature region, heterodimers are subsequently formed by slower chain exchange. If the temperature is too low for chain exchange to readily take place (below ≈25–30 °C), homodimers remain kinetically trapped. The preference for homodimer formation by refolding by dialysis or dilution at low temperature is similarly explained. Even with slow dialysis where equilibrium would appear to be attained, homodimers will preferentially be produced due to the very slow rate of chain exchange below room temperature. Cooling a mixture of separated chains from high temperatures slowly enough, however, would be expected to produce αβ. This explains the observation that the CD unfolding/refolding curves for αβ were reversible because the heating/cooling rate of 0.4 °C/min was slow enough to allow chain exchange to take place before the temperature was lowered to values where the exchange rate was too slow. In contrast to physiological salt solutions, in low salt solutions the CD unfolding profiles of the mixture of homodimers appeared normal, i.e. each homodimer unfolded independently without chain exchange. This may be expected in view of the unshielded charge repulsion of the negatively charged molecules in the neutral pH region resulting in a slower rate of chain exchange.

The observation that heterodimers are obtained in vitro from a mixture of homodimers by equilibrating at physiological temperature indicates that thermodynamics can explain the native αβ composition of GTm. The tendency for heterodimer preference over homodimers is greatest (Fig. 4A, curve 3). More definitive evidence for refolding to αβ in two steps was obtained by a repeat of the kinetic refolding studies at 40 °C, where the difference between the unfolding curves of homodimers and heterodimer is greatest (in Fig. 4A, compare curves 1 and 2). It is clearly seen that the negative ellipticity increased in two processes; a fast process which follows the temperature drop and a slow process with τs ≈600 s (Fig. 4B). It appears that the fast process is the production of homodimers in view of the smaller value of negative ellipticity initially obtained since αα is ≈50% unfolded at 40 °C. The slow process is chain exchange resulting in the production of αβ. Evidence for the fast process producing homodimers was also obtained by monitoring the unfolding profile of a sample which was cooled from 55 to 20 °C rapidly (0.3 °C/s). Although there was incomplete refolding during this fast temperature drop, it can be seen that an appreciable amount of homodimer was formed as evidenced by the presence of the truncated first transition (Fig. 4A, curve 3).

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1138

Smooth Muscle Tropomyosin Assembly

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Unfolding/refolding studies of smooth muscle tropomyosin. Evidence for a chain exchange mechanism in the preferential assembly of the native heterodimer.

S S Lehrer and Y Qian

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