FOR THE RECORD

Rapid, spontaneous reassembly of homo- and heterodimeric tropomyosin two-chain coiled coils from unfolded single α and β chains

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(RECEIVED September 8, 1992; REVISED MANUSCRIPT RECEIVED October 15, 1992)

Two-chain α -helical coiled coils constitute a very important subclass of proteins found in muscle, DNA-binding proteins (leucine zippers), and bacterial cell walls. Tropomyosin from rabbit muscle is a typical coiled coil, whose native state comprises two α -helical polypeptide chains in register and in parallel with a supertwist (Crick, 1953; McLachlan & Stewart, 1975). In their biological context, coiled coils are composed either of two identical chains (homodimers) or of two different chains (heterodimers). For example, tropomyosin from rabbit skeletal muscle is a mixture of α and β chains in a ratio between 3 and 4 to 1 (Lehrer, 1975). Both α and β chains have 284 residues but differ at 39 sites, mostly by conservative changes (Mak et al., 1980). In vivo, over 90% of the molecular species are $\alpha\alpha$ or $\alpha\beta$ dimers; indeed, it had long been accepted that no $\beta\beta$ species are present and that the in vivo molecular distribution is far from random (Eisenberg & Kielly, 1974; Lehrer, 1975; Strasburg & Greaser, 1976). Recently, however, such $\beta\beta$ species have been detected, and a distribution has been proposed that is much closer to random (Holtzer et al., 1992). Cellular preference for homodimer or heterodimer could be exercised through thermodynamic, kinetic, or biological control. Each mode of control has its advocates in investigations from various laboratories on tropomyosins from various sources (Bronson & Schachat, 1982; Holtzer et al., 1984; Lehrer & Qian, 1990).

Recent studies of tropomyosin by the stopped-flow technique and by high performance liquid chromatography (HPLC) suggest a new strategy by which to study the spontaneous recombination of two-chain coiled coils from unfolded α and β tropomyosin chains. Stopped-flow circular dichroism (SFCD) studies on rabbit tropo-

myosin show that the separated, unfolded chains (either α or β) self-assemble into the corresponding, intact homodimeric coiled coil in a few seconds or less under conditions strongly favorable to refolding (Mo et al., 1991a,b, 1992). Furthermore, it was found that the dimerization step and formation of $\frac{2}{3}$ of the equilibrium helix content occurs in a fast phase lasting less than 40 ms (Mo et al., 1991a). Therefore, unfolded single α and β chains can reassemble into dimers in the stopped-flow mixer by the sudden change of medium at controlled ($\leq 30^\circ\text{C}$) temperature, and the dimers can be disulfide crosslinked subsequently (after dialyzing out dithiothreitol [DTT]). It is known that the dimers do not exchange chains below 30°C (Holtzer et al., 1984), and that even at higher temperature, e.g., 36°C , exchange only occurs on a time scale of hours (Ozeki et al., 1991). Therefore, crosslinking simply fixes the existing homodimer–heterodimer population. The composition of the resulting crosslinked dimers can then be obtained by HPLC, because *crosslinked* dimers ($\alpha\alpha$, $\beta\beta$, and $\alpha\beta$) are well resolved by reversed-phase hydrophobic HPLC (Crimmins & Holtzer, 1991).

In this paper, we report the species composition of recombined dimers formed in the time scale of milliseconds from equimolar amounts of unfolded, separated α and β tropomyosin chains from rabbit skeletal muscle. To our knowledge, this is the fastest method of recombination of coiled coils thus far deployed. Previously, dilution by hand-mixing (a matter of seconds) (Crimmins & Holtzer, 1981; Brown & Schachat, 1985), quenching from higher temperature (seconds) (Holtzer et al., 1984; Brown & Schachat, 1985; Lehrer & Qian, 1990), and dialysis (hours) (Brown & Schachat, 1985; Graceffa, 1989; Holtzer et al., 1992) have been the methods (and time scales) employed.

Equimolar amounts of α and β chains were obtained from pure heterodimer $\alpha\beta$ as follows. Rabbit skeletal tropomyosin was crosslinked by ferricyanide (Holtzer et al.,

1986). The crosslinked $\alpha\beta$ dimer was separated from other species by reversed-phase hydrophobic HPLC using a semipreparative C_4 column and was characterized by analytical HPLC and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In the recombination experiments, the $\alpha\beta$ dimer was unfolded in urea_{6,000} NaCl₅₀₀ NaPi₅₀ (7.4) buffer and reduced by adding 20 mM DTT to the solution. We designate complex aqueous solvent media by giving each solute species a symbol or abbreviation with its millimolarity as subscript followed by the pH in parentheses. The protein solution was diluted 10-fold in the stopped-flow mixer at controlled temperature with the benign buffer sucrose₄₅₀ NaCl₅₀₀ NaPi₅₀ (7.4). The sucrose prevents mixing anomalies (Mo et al., 1991b). The assembly into coiled coils was completed in a few seconds, as judged by SFCD at 222 nm, the negative extremum of the α -helix backbone spectrum. Remaining urea, sucrose, and DTT were dialyzed out against the benign buffer NaCl₅₀₀ Tris₅₀ (7.4) at 4 °C. The protein was crosslinked by ferricyanide and dialyzed against deionized water exhaustively, followed by lyophilization. The dried sample was analyzed by reversed-phase hydrophobic HPLC with a C_4 analytical column. Details of our SFCD and HPLC methods have been published (Crimmins & Holtzer, 1991; Mo et al., 1991a,b; Holtzer et al., 1992).

An HPLC analysis is shown in Figure 1, and a summary of the results obtained for mixing at three temper-

Fig. 1. Reversed-phase high-performance liquid chromatograms for crosslinked rabbit skeletal tropomyosin (A), purified crosslinked $\alpha\beta$ species (B), and species reassembled by stopped-flow from unfolded, separated α and β chains at 10 °C and then crosslinked (C). Full scale is 1.6 absorbance units with offset 1.0, 0.6, and 0.1 for A, B, and C, respectively. Mobile phase A = 0.2% trifluoroacetic acid (TFA) in 25% aqueous acetonitrile and B = 0.2% TFA in 75% aqueous acetonitrile with a linear gradient from 40% to 85% B in 50 min at 1 mL/min. $\beta=\beta$ is doubly crosslinked species; $\alpha-\alpha$, $\alpha-\beta$, and $\beta-\beta$ are singly crosslinked species; and α and β are noncrosslinked species. In chromatogram C, $\beta=\beta$ = 21.3%, $\beta-\beta$ = 4.8%, $\alpha-\alpha$ = 25.9%, $\alpha-\beta$ = 43.8%, and α,β = 4.2%. Values averaged over all experiments are given in Table 1.

atures is shown in Table 1. The recombination involves three possible dimerizations: $\alpha + \beta = \alpha\beta$, $\alpha + \alpha = \alpha\alpha$, and $\beta + \beta = \beta\beta$. If the process is random, 50% $\alpha\beta$ and 25% each of $\alpha\alpha$ and $\beta\beta$ would result. On the other hand, if either heterodimer or homodimer forms preferentially, $\alpha\beta$ would be either more or less than 50% and $\alpha\alpha$ and $\beta\beta$ would each either be less or more than 25%. From Table 1, it is obvious that $\alpha\alpha$ and $\beta\beta$ each constitute near 25% and $\alpha\beta$ near 50% of the total sample. Therefore, when the unfolded α or β chains are brought to coiled coil-favoring conditions rapidly (<40 ms), no preference is given to formation of either homodimer or heterodimer. The fast recombination of α and β chains is random.

The experiments were carried out in media rather higher in ionic strength than physiological (to prevent tropomyosin aggregation). However, it has been shown (through use of “nonpolymerizable” tropomyosin) that lower ionic strength does not alter the kinetics of the dimerization step appreciably (Mo et al., 1991b). Hence, it is not likely that reducing the ionic strength in stopped-flow studies would alter the pattern of dimer species appreciably from that seen in Table 1.

In our stopped-flow kinetic studies on individual reassembly of homodimeric $\alpha\alpha$ or $\beta\beta$ tropomyosin, it was found that the dimerization is almost encounter-controlled in either case (Mo et al., 1991a, 1992). Furthermore, the difference between α and β chains is not very big, because most of the 39 mutations are conservative (Mak et al., 1980). In reassembly, either an α or a β chain probably will dimerize permanently with whatever chain it first encounters, regardless of whether it is the same as itself or not. Because in these experiments there are equal numbers of α and β chains, this random combination gives 50% of $\alpha\beta$ and 25% each of $\alpha\alpha$ and $\beta\beta$. This result, by itself, does *not* imply that the resulting random homodimer–heterodimer population is the equilibrium (thermodynamically controlled) one, although it might be. Once a given dimer forms at these temperatures, it is well known to be kinetically trapped (Holtzer et al., 1984). Thus, each chain is, as it were, obliged to remain with its first-encountered partner. An effect different in magni-

Table 1. Species composition after stopped-flow rapid reassembly of α and β tropomyosin chains^a

	10 °C	20 °C	30 °C
$\alpha\alpha$	25.1 ± 1.3	25.0 ± 1.2	24.8 ± 1.5
$\beta\beta$ ^b	26.0 ± 1.2	25.5 ± 1.5	26.7 ± 1.4
$\alpha\beta$	44.2 ± 1.1	45.3 ± 1.6	44.7 ± 1.9
α,β	4.6 ± 0.3	4.2 ± 0.2	4.0 ± 0.5

^a Total protein concentration in the refolding medium was 0.1 mg/mL. Values are percentages: mean ± SD for three stopped-flow reassembly runs for each temperature.

^b The sum of $\beta=\beta$ and $\beta-\beta$.

tude, but of similar sort, was observed some time ago in much cruder renaturation experiments on paramyosin, another coiled coil (Crimmins & Holtzer, 1981).

Acknowledgments

This work was supported in part by grant GM-20064 from the Division of General Medical Sciences, United States Public Health Services, and in part by a grant from the Muscular Dystrophy Association. A Wheeler Fellowship from Washington University to one of us (J.M.) is gratefully acknowledged.

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