Unfolding Domains in Smooth Muscle Myosin Rod†

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ABSTRACT: Gizzard smooth muscle myosin rod, an α-helical coiled coil, exhibits two cooperative thermal or denaturant-induced helix unfolding transitions in solutions containing 0.6 M NaCl at neutral pH, when monitored by circular dichroism at 222 nm. The first smaller transition unfolds part of the subfragment 2 (S2) domain, and the main transition unfolds the remaining helix including the light meromyosin (LMM) domain. These unfolding domains were identified by monitoring the fluorescence of acrylodan, an environmentally sensitive fluorescence probe, and the ESR signal of a maleimide spin-label, sensitive to motion, both specifically attached to Cys 43 in the S2 region of the rod sequence. The identities of the domains were verified by studying the unfolding of the S2 and LMM coiled-coil peptides obtained by proteolytic cleavage of spin-labeled and unlabeled rod. The fluorescence of acrylodan-labeled rod indicated that although the S2 intermediate is unfolded, it is not in a random-coil conformation The unfolded S2 region stabilized the LMM domain against unfolding, possibly by a direct interaction with the LMM region. Such an interaction may be involved in the salt- and phosphorylation-dependent 6S to 10S shift in configuration of the myosin molecule.

Smooth muscle myosin is composed of 2 heavy chains each containing 1979 amino acid residues (Yanagisawa et al., 1987). The N-terminal 849 amino acid residues of each heavy chain, together with 2 light chains, form a globular head (S1). Each head contains an ATP binding site, an actin binding site, and two light chains. Phosphorylation of one of the light chains, the regulatory light chain, is the primary control of ATPase activity and motility (Sobieszek, 1977; Sellers et al., 1985). The C-terminal 1130 amino acid residues of each heavy chain combine to form a coiled-coil α-helix. This rod region is involved in the assembly of myosin into thick filaments, the functional form of myosin in muscle. The rod can be separated from the heads by limited papain proteolysis (Sobieszek & Small, 1976). Subsequent digestion of the rod with either trypsin or chymotrypsin cleaves it into two subfragments: S2, the portion immediately adjacent to the head, and LMM, the C-terminal two-thirds portion of the rod (Cross et al., 1984; Suzuki et al., 1984). A single cysteine amino acid residue is located at position 43 from the head−rod junction (Yanagisawa et al., 1987) and has been shown to react rapidly with *N*-ethylmaleimide (Nath et al., 1986).

Smooth muscle myosin has been shown to undergo a phosphorylation-dependent configuration change from a 6S sedimenting species to a "folded-up", 10S sedimenting species (Onishi & Wakabyashi, 1982; Trybus et al., 1982). Electron micrographs of the 10S species indicated that the rod can "bend" in two places and interact with the part of S2 near the head−rod junction (Onishi & Wakabyashi, 1982; Trybus et al., 1982). This suggests that the rod is able to unfold locally, allowing these interactions to take place.

In this work, Cys 43 in the N-terminal part of S2 of the rod was labeled with a fluorescence probe, acrylodan, and a maleimide spin-label, MSL. The thermal and GdmCl-induced helix unfolding was monitored by CD, fluorescence, and ESR. Two cooperative helix unfolding transitions were observed, a smaller pretransition, involving part of the S2 domain, and a main transition, involving the LMM domain. From the fluorescence properties of the acrylodan probe, it appears that the partially unfolded intermediate has the properties of a condensed coil whereby the probe is partially shielded from the solvent. Comparison of the unfolding of the S2 and LMM regions in intact rod with the unfolding of purified S2 and LMM peptides indicated that the unfolded S2 domain in the rod stabilized the LMM domain. The ability of S2 to stabilize LMM suggests an interaction between the two regions which may be of importance in the 6S to 10S transition. A preiminary report has been presented (King et al., 1989).

EXPERIMENTAL PROCEDURES

Chicken gizzard myosin was prepared by the method of Ebashi et al. (1976). Ammonium sulfate fractionation and column chromatography were used to substitute for the several high-salt−low-salt polymerization cycles. To prepare rod, myosin (5 mg/mL) in 0.6 M NaCl, 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, (high-salt buffer), and 1 mM dithiothreitol was digested with 25 ug/mL activated papain for 45 min at room temperature and the digestion terminated by 10 mM iodoacetic acid and immediately dialyzing the digestion mixture against 40 mM NaCl, 20 mM sodium phosphate, 1 mM EDTA, and 1 mM dithiothreitol, pH 6.5 (precipitation buffer), in the presence of 1 mM phenylmethanesulfonyl fluoride. This dialysis buffer was

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changed initially after 1−2 h and several times during the dialysis. Papain used for digestion (1 mg/mL) was activated for 1 h at 37 °C in a solution containing 50 mM of freshly dissolved cysteine, 10 mM EDTA, and 10 mM Tris, pH 8. Rod was obtained by centrifugation at 100000*g* for 90 min. The pellet was dissolved in high-salt buffer for further purification with Bio-gel A-15 column chromatography, and fractions of pure rod identified by SDS−PAGE were collected and concentrated for fluorescence and spin-labeling.

To prepare isolated LMM and S2, rod at 6 mg/mL in 0.5 M NaCl, 10 mM MOPS buffer, pH 7, was digested with 5 ug/mL α-chymotrypsin (Worthington, Freehold, NJ) at 25 °C for 40 min, quenched with phenylmethanesulfonyl fluoride, dialyzed vs precipitation buffer, and centrifuged as for rod preparation, above. S2 was obtained in the supernatant, LMM in the pellet. SDS−PAGE indicated less than 10% contamination of LMM and S2. Spin-labeled S2 was obtained by digestion of labeled rod with this procedure.

For labeling with the environmentally sensitive probe acrylodan (Weber & Farris, 1979; Prendergast et al., 1983; Marriott et al., 1988; Lehrer & Ishii, 1988), myosin rod was reduced with 50 mM dithiothreitol at room temperature for 2 h in high-salt buffer to ensure complete reduction of possible disulfide bonds, followed by exhaustive dialysis to remove the dithiothreitol. The labeling reaction was started immediately after the dialysis so as to minimize reoxidation of the rod sample from occurring. The labeling reaction was carried out in high-salt buffer with a 5:1 molar ratio of acrylodan (Molecular Probes, Eugene, OR) to rod for 1 h at room temperature. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) blocked myosin rod was used as a control and was treated with acrylodan identically to the unblocked sample (Lehrer & Ishii, 1988). Lack of appreciable acrylodan label incorporation indicated that acrylodan reacted specifically with Cys groups. Some minor labeled impurities, as well as unreacted acrylodan, were removed by Bio-gel A-15 gel filtration. The fractions of pure acrylodan-labeled rod identified by the fluorescent band on polyacrylamide gel were collected for fluorescence studies. The degree of labeling was estimated to be 0.4−0.7 acrylodan/chain from absorption spectra.

For spin-labeling, reduced rod in precipitation buffer containing 0.1 mM dithiothreitol was taken up in high salt buffer at 7−8 mg/mL in 0.5 M NaCl, 130 mM MOPS buffer, pH 7.5, labeled with 0.15 mM MSL (3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy; Syva, Palo Alto, CA) for 30 min at room temperature, and dialyzed vs 0.5 M NaCl, 10 mM MOPS buffer, pH 7.5.

Circular dichroism measurements were made with an Aviv 60 DS spectropolarimeter (Lakewood, NJ) containing a Hewlett-Packard 89100A temperature controller which provided programmable sample temperature changes. Ellipticity values at 222 nm were obtained from 25 to 70 °C using solutions in a stoppered standard 1-cm Spectrosil quartz cuvette into which the temperature probe of the unit was placed. The solution was stirred with a magnetic bar placed below the light path, enabling rapid temperature equilibration to be obtained. Data were collected automatically in 0.2 °C steps using an equilibrium time of 0.4 min and a data averaging time of 10 s at each temperature step. The protein concentration was 0.05−0.1 mg/mL. The sample temperature is accurate to within 0.5 °C.

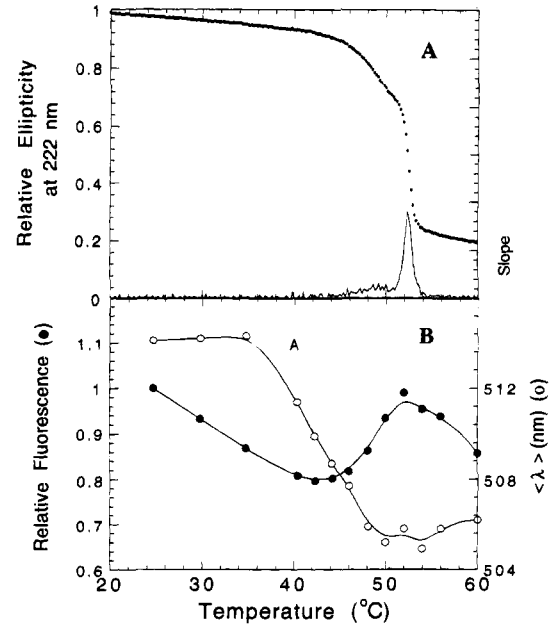

FIGURE 1: Temperature dependence of the helix unfolding profile and acrylodan fluorescence of chicken gizzard myosin rod. (A) Normalized ellipticity at 222 nm (upper curve) and slope (lower curve). (B) Relative quantum yield (●) and weighted average spectral position of acrylodan fluorescence (○). Rod in 0.6 M NaCl, 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, and 1 mM dithiothreitol.

Fluorescence measurements were carried out with an ISS K-2 fluorometer (Urbana, IL) in the ratio mode excited at 375 nm. The weighted average fluorescence wavelength was calculated from 400 to 660 nm in 1-nm steps as described earlier (King & Lehrer, 1989). The relative fluorescence quantum yield was calculated as the area under the spectrum over the same wavelength range. The time dependence of fluorescence intensity was carried out with a photon counting Spex Fluorolog 2/2/2 spectrofluorometer (Edison, NJ) in the ratio mode with the same excitation and emission wavelengths. Protein concentrations were 0.025−0.04 mg/mL. The sample temperature is accurate to within 1 °C, and the average maximum wavelength is accurate to within 1 nm.

The electron spin resonance measurements were obtained with a Varian E-109I ESR spectrometer operating at 9.5 GHz. A sample cell of 0.1 mL volume was temperature-regulated with the Varian V-9540 temperature controller and determined with a miniature thermocouple attached to the flat surface of the cell. Protein concentrations were 1−2 mg/mL.

RESULTS

Fluorescence Changes Associated with Thermal Unfolding of Rod. The circular dichroism (CD) spectrum of gizzard myosin rod has ellipticity peaks at 222 and 208 nm characteristic of an α-helix similar to previous spectra obtained in CD studies with skeletal rod (King & Lehrer, 1989). The temperature dependence of the rod ellipticity at 222 nm exhibits two unfolding transitions, a pretransition and a main transition with midpoints at 48 and 52 °C, respectively (Figure 1A), in buffer containing 0.6 M NaCl. About 20−25% and about 50% of the rod helix are unfolded in the pretransition and main transitions, respectively. This suggests that much of the S2 region may unfold as a domain in the pretransition since it is about half the size of the LMM

region. Transition 2 is highly cooperative, being complete within 2 °C. About 20% of the ellipticity remains at 60 °C. There was some indication of time-dependent aggregation at the higher temperatures and higher concentrations (see below). A control unfolding study at 2× the heating rate used in Figure 1 gave identical unfolding profiles with >90% reversibility. At higher concentrations than the 0.05−0.1 mg/mL used in these CD runs, the reversibility was less. Thus, the small degree of aggregation did not affect the helix unfolding under the conditions reported here.

The thermal unfolding profile of acrylodan-labeled rod is the same as that of unlabeled rod, indicating that the covalent attachment of acrylodan at Cys 43 does not appreciably perturb the protein structure. To determine which transition involves the S2 region, the fluorescence properties of labeled rod were studied. Both the relative fluorescence quantum yield (integrated intensity) and the emission spectrum were sensitive to the thermal unfolding. A change in quantum yield reflects a change in quenching by local groups by solvent, while the spectral position is sensitive to the exposure of the fluorophore to solvent. In the temperature region up to 40 °C, the relative quantum yield monotonically decreased, due to thermally activated quenching processes (Figure 1B). Between about 44 and 50 °C there was an increase in intensity and a large blue spectral shift which correlate with the pretransition in the helix unfolding profile and were essentially complete prior to the main transition. These results indicate that a domain in the S2 region containing the acrylodan is the least stable region of the rod.

To obtain information about some aggregation which appeared to occur at the higher temperatures, the kinetics of the fluorescence change were compared to the light-scattering change, a measure of aggregation. After an immediate increase of temperature from 25 to 48 °C, there was an initial change in fluorescence intensity due to the temperature change accompanied by a small blue shift in fluorescence due to an environmental change, followed by a slow increase in intensity in parallel with a further blue shift. The light scattering increased over a long period of time, indicating that the slow fluorescence spectral blue shift and intensity increase were due to aggregation (Figure 2). However, the time for 50% scattering intensity change was about 20 min while the time for 50% fluorescence change was less than 10 min, indicating that the fluorescence change preceded the light-scattering increase. It appears that there is a local unfolding which occurs in the pretransition resulting in a blue spectral shift followed by an aggregation process.

Fluorescence Changes Associated with Denaturant-Induced Unfolding. The GdmCl concentration dependence of the ellipticity at 222 nm showed three unfolding transitions: a pretransition with a midpoint at 1.2 M GdmCl corresponding to a loss of about 15% total helix, a major cooperative transition with a midpoint at 1.7 M GdmCl corresponding to the loss of about 60% total helix, and a broad posttransition with a midpoint at 3.5 M GdmCl in which the remaining helix was lost (Figure 3A). In view of the similarity to the thermal helix unfolding profile, it appears that the pretransition and main transitions involve similar unfolding domains of the rod. In contrast to thermal unfolding, however, where appreciable ellipticity remained at high temperature, very little ellipticity was left at high GdmCl concentration.

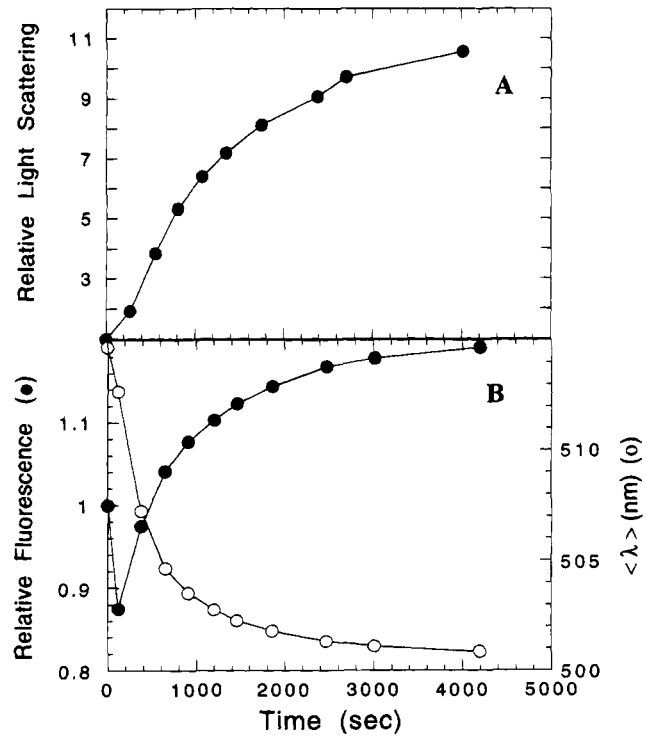

FIGURE 2: Kinetics of the acrylodan fluorescence change associated with the unfolding of rod in the thermal pretransition. At $t = 0$, the temperature was increased to 48 from 25 °C. (A) Increase in light scattering. (B) Relative quantum yield (●) and weighted average emission wavelength (○) of acrylodan fluorescence. Solution conditions as for Figure 1.

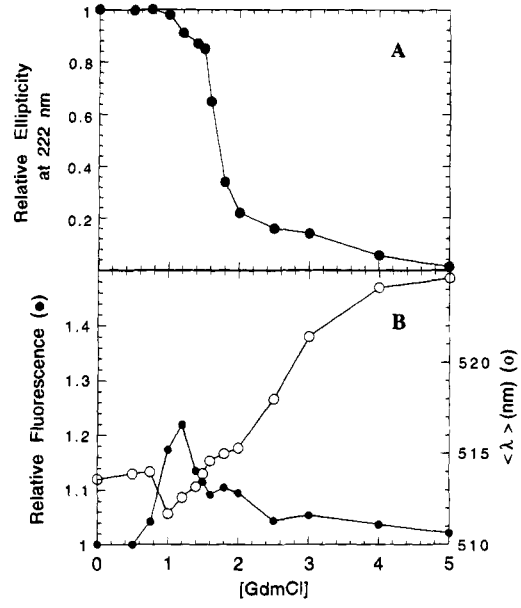

FIGURE 3: GdmCl dependence of the helix unfolding profile and acrylodan fluorescence of chicken gizzard myosin rod. (A) Normalized ellipticity at 222 nm. (B) Relative quantum yield (●) and weighted average emission wavelength (○) of acrylodan fluorescence.

Changes in acrylodan fluorescence properties were associated with each helix transition. A 2.5 nm blue shift and 22% intensity enhancement were associated with the pretransition. In the major helix transition, both fluorescence properties shifted back toward the values in the absence of GdmCl. There was approximately 10 nm of spectral shift toward the red in the posttransition without much fluorescence intensity change. The fluorescence changes associated

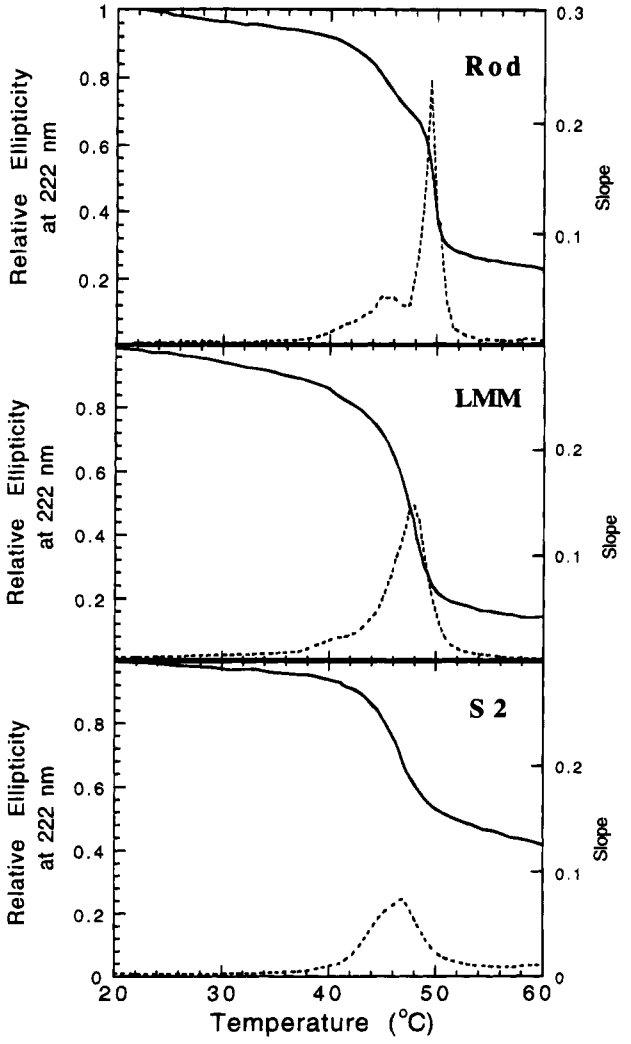

FIGURE 4: Temperature dependence of the helix unfolding profiles of chicken gizzard rod compared to its proteolytic peptide fragments, LMM and S2. Solution conditions: 0.5 M NaCl, 5 mM sodium phosphate buffer, pH 7.0, 0.1 mM EDTA, and 1 mM dithiothreitol.

with denaturant unfolding are different from those of thermal unfolding. The principal difference is that although in both cases a shift in the spectrum toward the blue was observed in the pretransition, suggesting a decrease in exposure to solvent, there were further shifts in the spectrum toward the red only for the denaturant-induced unfolding, associated with greater probe exposure to solvent. The lack of blue spectral shift in the main thermal transition indicates that temperature-dependent aggregation is inhibited in the Gd-mCl-induced unfolding.

Comparison of Unfolding Profiles of Rod with LMM and S2. The thermal unfolding profile of rod in 0.5 M NaCl showed a pretransition at 46 °C and a main transition at 49–50 °C, very similar to that in 0.6 M NaCl, except for a 2 °C shift of the unfolding profile to lower temperature (Figure 4). The LMM peptide, which makes up 68% of the C-terminal sequence of the rod, had a single transition at 48 °C which was much broader than rod's main unfolding transition. The S2 peptide, which is composed of 32% of the N-terminal sequence of rod, showed a broad transition at 46 °C. From the shape and magnitude of the unfolding profile of S2, most of the S2 region of the rod appears to be responsible for the pretransition in the rod unfolding profile. Although the LMM portion in the rod unfolds in the main

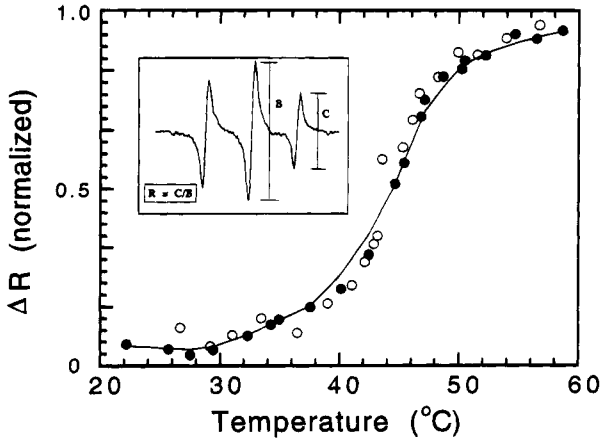

FIGURE 5: Temperature dependence of the relative mobility of maleimide nitroxide spin-labeled rod (○) and S2 (●) determined by electron spin resonance. Insert: ESR spectrum showing the definition of the mobility parameter, *R*. Proteins at 1.9 mg/mL in 0.5 M NaCl, 5 mM sodium phosphate, pH 7.0, and 0.1 mM EDTA.

transition, the transition is more cooperative and occurs at somewhat greater temperature than LMM alone, indicating an influence of the unfolded S2 region in the rod on the LMM unfolding. S2 exhibits appreciable 222 nm ellipticity at high temperature which appears to explain the 20–25% ellipticity of rod remaining at 60 °C.

Further evidence for the S2 portion of the rod unfolding in the pretransition was obtained by monitoring the temperature dependence of the ESR signal of a maleimide nitroxide spin-label at Cys 43 in rod and in S2 (Figure 5). It can be seen that the mobility of the spin-label increased in a cooperative transition with a T_m = 45 °C with identical temperature dependence for both systems, rod and S2.

DISCUSSION

Unfolding Domains in Gizzard Rod. The main finding in this work is that the coiled-coil α-helix of gizzard rod consists of at least two cooperative unfolding domains corresponding to the S2 and LMM regions. The S2 region is the less stable domain since the properties of both a fluorescence and a spin probe labeled at Cys 43 change over the same temperature region and denaturant concentration as a small transition (pretransition) which occurs prior to the main unfolding transition. A previous differential scanning calorimetry study only showed one thermal transition for gizzard muscle rod at a similar temperature as the main transition reported here (Cross et al., 1984). These results on gizzard rod should be contrasted with studies with rabbit skeletal myosin rod which experiences multiple thermal unfolding transitions involving several regions of different stabilities (Burke et al., 1973; Goodno et al., 1976; Privalov, 1982; Cross et al., 1984; Stafford, 1985; King & Lehrer, 1989).

The S2 domain unfolds independently of the LMM domain since the unfolding profile of the S2 domain in the intact rod compared to the S2 coiled-coil peptide and the mobility change of the spin-label in the S2 peptide compared to intact rod were all very similar. The LMM domain, however, is somewhat more stable and unfolds with temperature more cooperatively in the rod than in the isolated LMM coiled-coil peptide. This suggests that there is a stabilizing interaction between the unfolded S2 domain and the folded LMM domain of the rod. Aggregation takes place at temperatures in the pretransition, presumably involving

hydrophobic regions of unfolded S2, as has been observed on removing the regulatory light chain from myosin (Trybus & Lowey, 1988). However, aggregation was a slow process and at the heating rate employed in this study does not appear to cause the stabilization. Although aggregation does not occur for denaturant-induced unfolding, it is not clear if the denaturant-induced main transition is also more cooperative, because of the fewer data points obtained in the transition. There are two possible mechanisms to explain the stabilizing influence of S2: (i) surviving structure in unfolded S2 forming a noncovalent interchain cross-link; (ii) direct interaction between unfolded S2 and the LMM domain. A disulfide cross-link has been shown to stabilize the coiled-coil tropomyosin molecule (Lehrer, 1978), and a stable domain consisting of interacting N-terminal fusion peptides stabilizes the N-terminal domain of fusion tropomyosin (Ishii et al., 1992). The unfolded S2 domain could directly interact with a part of the LMM region. This direct interaction could be part of the mechanism for the interaction of the bent rod with the S2 region at the head−neck junction involved in the 6S to 10S transition. The salt concentrations required for the transition are lower than used here, possibly allowing the S2 domain to unfold at lower temperature. Further studies are planned to determine if this direct interaction is occurring.

In addition to the pretransition and the main transition, the denaturant-induced helix unfolding profile of the rod exhibited a posttransition between GdmCl concentrations from 2 to 5 M, resulting in the complete loss of 222 nm ellipticity signal of rod producing a putative random coil. In contrast to GdmCl-induced unfolding, thermal unfolding leaves about 25% ellipticity signal at high temperature, further evidence for hydrophobic interactions maintaining some structure. This may be mainly due to the S2 region, since 40% ellipticity remains at 60 °C for the S2 peptide in contrast to 20% for the LMM peptide.

Nature of the Unfolded Intermediates. There is a fluorescence blue shift and intensity enhancement associated with the pretransition with somewhat smaller changes in these properties for the GdmCl-induced pretransition compared to the thermal pretransition. A red spectral shift is expected for acrylodan attached to a peptide in a random-coil conformation with increased exposure to solvent. The lack of a red shift in this system further extends the observation often found of the non-random-coil nature of partially unfolded intermediates of coiled-coils (Betteridge & Lehrer, 1983; King & Lehrer, 1989; Ishii et al., 1993; Mo et al., 1991). The blue shift indicates that the acrylodan environment becomes less polar; i.e., the unfolded region of the intermediate appears to present a hydrophobic environment to shield the acrylodan from water. In the main denaturant-induced transition, the acrylodan experiences a red shift, indicating that the unfolding of the rest of the molecule disrupts the structure of the unfolded S2 region to expose the acrylodan probe to solvent. For the thermal unfolding, a red spectral shift was not experienced due to the time-dependent aggregation process which prevented exposure of the probe. Thus, the thermal unfolding of the S2 domain results in a quick blue fluorescence spectral shift while the time-dependent aggregation process produces further shifts to the blue.

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