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Effects of Interchain Disulfide Cross-Links on the Trypsin Cleavage Pattern and Conformation of Myosin Subfragment 2†

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ABSTRACT: The ability of 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) to produce interchain disulfide cross-links in both the long and short forms of myosin subfragment 2 (S2) and the conformational effects of these cross-links have been investigated. Short S2 (residues 3–287) contains two pairs of Cys residues at positions 66 and 108, and long S2 (residues 1–440) contains an additional pair at position 410. The reaction kinetics of each form of S2 with Nbs_2 was biphasic. During the fast kinetic phase the reaction resulted in un-cross-linked species having Nbs-blocked Cys. During the slow phase disulfide-cross-linked species were formed via interchain S-Nbs/SH exchange. For short S2, Cys-66 appeared to react without forming disulfide cross-links, and the Cys-108 pair reacted with partial cross-linking. For long S2, the Cys-66 pair appeared to react with partial cross-linking, and the Cys pairs at 108 and 410 reacted with complete cross-linking. Mild tryptic digestion of disulfide-cross-linked long S2, under conditions that resulted in partial production of short S2 from un-cross-linked LS2, produced peptides T_{1a} and T_{1b} (residues 1 to ~360), with one and two disulfide cross-links, respectively. Further digestion of cross-linked long S2 or cross-linked short S2 resulted in the same shorter fragment, T_2, with an NH_2-terminus beginning at 103 consistent with a sequence of residues 103–287. Circular dichroism studies on long S2 indicated that the presence of disulfide cross-links changed the thermal unfolding profile of the helix. A destabilizing pretransition was observed between 25 and 40 °C for the cross-linked long S2. These studies indicate that interchain disulfide cross-links can create new proteolytic cleavage sites located far from the cross-links as a result of increased destabilization.

Each of the two heavy chains of the myosin molecule consists of an N-terminal globular head portion [S1, M_r(chain) ~100 000] that contains the adenosinetriphosphatase (ATPase) and actin binding sites and a rodlike coiled-coil portion [rod, M_r (chain) ~130 000], the C-terminal part of which [LMM,[1] M_r (chain) ~70 000] forms the core of the thick filament. Most current models of muscle contraction associate the force generation with one or more states which differs in the disposition of the heads relative to the rod (Huxley, 1969; Huxley & Simmons, 1971; Eisenberg et al., 1980). Another force-generating mechanism which has been suggested involves a conformational change in the S2 portion of the rod, i.e., the portion between the heads and LMM (Harrington, 1971, 1979). Localized helix–coil transition (melting) in the rod near the LMM–S2 junction has been interpreted as supporting this concept (Tsong et al., 1983; Swenson & Ritchie, 1980).

Studies with tropomyosin, the prototype of a coiled-coil α-helical molecule, have produced evidence for regions of localized melting (Woods, 1969, 1976; Satoh & Mihashi, 1972; Chao & Holtzer, 1975; Lehrer, 1978; Graceffa & Lehrer, 1980; Potekhin & Privalov, 1982; Betteridge & Lehrer, 1983). The extent of the localized unfolding has been shown to increase upon the introduction of an interchain disulfide bond possibly as a consequence of steric strain (Lehrer, 1975, 1978). This increased unfolding in disulfide-cross-linked tropomyosin appears to be related to an increase in tryptic cleavage at certain sites (Gorecka & Drabikowski, 1977; Ueno, 1984).

Two forms of S2, long S2 (LS2) and short S2 (SS2), can be prepared by proteolytic digestion of myosin (Weeds & Pope, 1977; Highsmith et al., 1977; Sutoh et al., 1978). LS2 spans the whole length between the head/rod junction and the heavy meromyosin/LMM junction, whereas SS2 includes two-thirds of the N-terminal portion of LS2 (Lu, 1980). In the present work we studied the reaction of Nbs_2 with both forms of S2, and we found that disulfide cross-links were produced in both cases. Furthermore, as a result of these cross-links, trypsin produced a different pattern of cleavage. For disulfide-cross-linked LS2, the initial cleavage site near residue 360 and the new secondary cleavage site at Arg-102 are probably due to the presence of disulfide bonds at positions 410 and 108, respectively. Circular dichroism studies of LS2 in the 25–40 °C temperature range indicated an increased unfolding pretransition when the cross-links were present. These studies

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[1] Abbreviations: Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); SS2, short subfragment 2; LS2, long subfragment 2; LMM, light meromyosin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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indicate that localized perturbations in these coiled-coil structures can result in structural changes some distance away from the perturbation.

Experimental Procedures

Short S2 was prepared by tryptic digestion (TRTPCK; Worthington) of heavy meromyosin, prepared from myosin isolated from rabbit back muscle, as described earlier (Lu, 1980). A trypsin to substrate ratio of 1:75 w/w at 21 °C was used in a solution containing 30 mM Tris-HCl, pH 8.0, and 2 mM EDTA for 45 min. Excess soybean trypsin inhibitor (2 × weight of trypsin used) (Worthington) was added to quench the cleavage reaction.

Long S2 was prepared by digesting myosin red with a trypsin to substrate ratio of 1:400 w/w in a solution containing 0.5 M KCl and 20 mM sodium phosphate, pH 6.5, for 45 min at 21 °C, and excess soybean trypsin inhibitor was added to quench the reaction, as described earlier (Lu, 1980).

For kinetic studies with Nbs_2, LS2 and SS2 were further purified by isoelectric precipitation and reduced by treatment with dithiothreitol. The pH was reduced to 4.5 with acetic acid, and the pellet was separated and redissolved at pH 7.5 (20 mM Hepes, 1.0 M NaCl, and 1 mM EDTA) and reduced with 20 mM dithiothreitol for $^1/_2$ h at 35 °C and 48 h at 0 °C followed by gel filtration through a G-25 column equilibrated with 2.0 mM Hepes buffer, pH 7.5, and 1.0 mM EDTA to remove the excess dithiothreitol. The concentrations of the protein solutions were determined by the Lowry method using tropomyosin as a standard.

For preparing Nbs_2-cross-linked SS2 or LS2, the proteins were treated with 1 mM Nbs_2 in a solution containing 10 mM Hepes, pH 7.5, 50 mM NaCl, and 1 mM EDTA, for 30 min at 21 °C, followed by extensive dialysis vs. the same buffer to remove the excess Nbs_2.

Tryptic peptides of Nbs_2–LS2 were prepared for Edman degradation by digesting Nbs_2–LS2 with trypsin for 30 min at 21 °C with an enzyme to substrate ratio of 1:50 w/w to obtain T_{1a} and T_{1b} (see Results) or an enzyme to substrate ratio of 1:40 to obtain T_2 (see Results). The reaction was stopped with excess soybean trypsin inhibitor, and the mixture was lyophilized. Small peptides, trypsin, and trypsin inhibitor were removed by passage of the sample through a reversed-phase C_{18} column on a Beckman HPLC system. A gradient of 0–60% CH_3CN in 0.1% trifluoroacetic acid was used for eluting the peptides. Various fractions were examined on SDS–PAGE, and those containing T_{1a} + T_{1b} or T_2 were combined and subjected to Edman degradation.

To label the Cys of T_2 with iodo[^{14}C]acetic acid (New England Nuclear), T_2 was first reduced with dithiothreitol and then alkylated with 10-fold molar excess of radioactive iodoacetic acid. The reaction mixture was dialyzed vs. deionized water extensively and ^{14}C-labeled T_2 was recovered in the precipitate.

The Edman degradations were carried out on a Beckman 890C sequenator using program 03179. Polybrene was routinely added to the peptides before sequencing (Tarr et al., 1978). The phenylthiohydantoin derivatives of amino acids were identified by thin-layer chromatography (Laursen, 1971) and by amino acid analysis after regeneration of free amino acids by hydrolysis in 56.6% hydriodic acid at 150 °C for 5 h (Smithies et al., 1971). Amino acid analyses were performed on a Beckman 119 CL analyzer. In the case of ^{14}C-labeled T_2, aliquots of the phenylthiohydantoin derivatives were counted on a Beckman 7500 scintillation counter using a xylene-based scintillation solution (Anderson & McClure, 1973).

The kinetics of reaction of reduced SS2 and LS2 were followed at 412 nm on a double-beam Perkin-Elmer lambda 3 thermostated spectrophotometer. The reactions were carried out in 1.0-cm path-length semimicrocuvettes with 1.0 mL of sample containing 0.2–0.3 mg/mL SS2 or LS2 and an identical volume of buffer in the reference cuvette. The reaction was initiated by mixing 10 μL of 0.1 M Nbs_2 with the sample solutions after adding an equivalent amount to the reference cuvette. The total absorbance change was in the range 0.1–0.2. Ten to twenty data points of $A_\infty - A_t$ vs. time were read from the charts and computer fitted (PDP 11-44) by a nonlinear least-squares routine to a two-exponential scheme, $(A_\infty - A_t)/A_\infty = f_1 \exp(-k_1 t) + f_2 \exp(-k_2 t)$, programmed by Dr. T. Scott, in our department.

Circular dichorism measurements were performed on a computer-controlled updated Cary 60 (Aviv Associates, Lakewood, NJ), on 0.26 mg/mL solutions of LS2 or Nbs_2–LS2 in 0.6 M NaCl, 0.1 mM EDTA, 5 mM sodium phosphate buffer, pH 7.3, in a 1-mm thermostated cylindrical cuvette. The LS2 solution also contained 5 mM dithiothreitol. The ellipticity at 222 nm was recorded for about 1 min on a time base recorder at about 5 °C intervals after equilibrating for about 15 min at each temperature. The temperature of the solution was determined with a calibrated thermocouple (Omega Engineering, Stamford, CT).

SDS–PAGE analyses were carried out with either a homemade low-resolution minigel apparatus or a high-resolution commercial apparatus (Hoeffer, Bio-Rad). Minigels (0.25 × 4 × 8 cm) were made of 7.5% acrylamide and run in 0.1 M Tris–bicine buffer. The high-resolution system consisting of 9.5% acrylamide and 0.25% methylenebis(acrylamide) was run with a Laemmli stacking buffer system (1970).

Results

Location of Cys in the Sequence of S2. Sequence analyses of rod, SS2, and LS2 have shown that the NH_2-terminus of LS2 begins at residue 1 and ends at residue 440 of the rod whereas the sequence of SS2 begins at residue 3 and ends at residue 287 of the rod (Lu & Wong, 1985). A Cys found in the sequence of SS2 (Capony & Elzinga, 1981) can therefore be placed at position 108 of both SS2 and LS2 and a Cys found in the region of the sequence between LS2 and SS2, at position 410 (Lu & Wong, 1985). Since SS2 and LS2 contain two and three Cys, respectively, there is a second Cys between residues 1 and 287 (Lu, 1980). To determine the position of this Cys, LS2 was alkylated with iodo-[^{14}C]acetic acid and digested extensively with trypsin, and three ^{14}C-labeled peptides were isolated by HPLC techniques (Pliszka and R. C. Lu, unpublished results). Two peptides were found that contained Cys corresponding to the positions described above and a third peptide with the following sequence: Cys-Asp-Gln-Leu-Ile-Lys. This Cys-containing peptide has been previously reported by Kimura & Kielley (1966) and Weeds & Hartley (1968). The sequence of SS2 reported by Capony & Elzinga (1981) contained a sequence identical with the above peptide except for a Gln at the Cys position (residue 66). It appears that Cys had been mistaken for Gln. This is understandable since an iodoacetamide-modified N-terminal Cys can cyclize with its amino group and become refractory to Edman degradation in an analogous fashion to the formation of a pyrrolidone ring of a Gln residue at the N-terminus (Doolittle, 1972). In view of these new data we can now locate the second Cys in the 1–287 stretch at position 66. Thus, SS2 contains two Cys located in the amino-terminal half of the peptide, and LS2 contains an additional Cys located about one-tenth of the

Table I: Pseudo-First-Order Constants (k_1 and k_2), Fractional Contributions (f_1 and f_2), and Apparent Second-Order Rate Constants (k'_1 and k'_2) for the Reaction of SS2, LS2, and Tropomyosin (TM) with Nbs_2 at pH 7.5

system	T (°C)	f_1	k_1 (min^{-1})	f_2	k_2 (min^{-1})	k'_1 (M^{-1} s^{-1})	k'_2 (M^{-1} s^{-1})
SS2[a]	16	0.63	1.1	0.37	0.052	16	0.77
LS2[a]	16	0.22	1.2	0.78	0.039	18	0.58
TM[b]	16			1.00	0.044		0.70
SS2[a]	25	0.68	3.0	0.32	0.158	45	2.35
LS2[a]	25	0.31	1.7	0.69	0.161	30	2.39
BME[c]	25					1200	

[a] Measured in 1.8 mM Hepes buffer, pH 7.5, 1 mM EDTA, and 0.5 M NaCl with 1.12 mM Nbs_2. [b] Measured in 5 mM phosphate buffer, pH 7.3, 1 mM EDTA, and 0.6 M NaCl where k_2 (obtained) = 0.026 m^{-1} which was corrected for pH (Malthouse & Brocklehurst, 1980). [c] 2-Mercaptoethanol values obtained from Malthouse & Brocklehurst (1980).

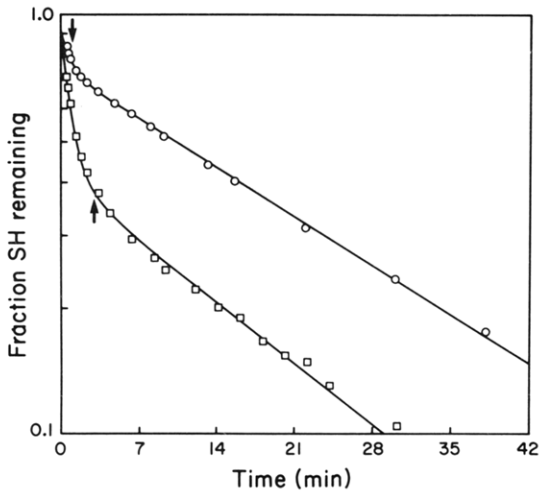

FIGURE 1: Kinetics of reaction of SS2 and LS2 with Nbs_2 at 16 °C. SS2 (□); LS2 (○). Arrows indicate times where 0.5% SDS was added in separate runs to quench any cross-linking reactions. Conditions were the same as in Table I.

length from its carboxyl terminus. No Cys residues have been found in the corresponding region of nematode myosin (McLachlan & Karn, 1982).

Reaction of LS2 and SS2 with Nbs_2. The rate of the reaction of the SH groups of LS2 and SS2 with excess Nbs_2 at 16 and 25 °C was monitored at 412 nm (due to the production of Nbs^-) until no further change occurred. The total number of SH/S2 chain obtained from the total absorbance change was 1.7 ± 0.2 for SS2 and 2.5 ± 0.2 for LS2, in agreement with the amino acid sequence data which shows 2 and 3 Cys/chain for SS2 and LS2, respectively (see above). The pseudo-first-order kinetic plots at both temperatures for both peptides were biphasic, the second reaction being 10–30 times slower. Examples of the data and curves that were computer fitted to the data assuming two independent rates are shown in Figure 1. The rate constants and associated fraction of SH groups obtained from the fitting routines for SS2, LS2, and tropomyosin under different conditions are shown in Table I. The rate constants for the corresponding phases of both peptides were about the same. For SS2, about 65% and 35% of the Cys are involved in the fast and the slow reaction with Nbs_2, respectively. For LS2, the corresponding values were 25% and 75%. If one pair of SH groups in each peptide reacted during the fast phase and one pair of SS2 and two pairs of LS2 reacted during the slow phase, the percent reacting in the fast phase would be 50% for SS2 and 33% for LS2. The actual percentages suggest that the kinetics are somewhat more complicated. To obtain further information about the reaction, gels of the Nbs_2-reacted peptides were run after the reaction was completed. About 20–40% of SS2 was cross-linked (Figure 2, slot F), indicating that neither of the two Cys pairs were completely cross-linked. For LS2, >95% was crosslinked,

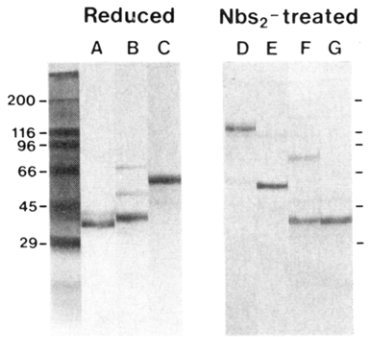

FIGURE 2: SDS gels of LS2 and SS2 before and after reaction with Nbs_2. Left, lanes A–C run in the presence of DTT; right, lanes D–G run in the absence of DTT. Left slot: globular protein molecular markers in $M_r \times 10^{-3}$. A, rabbit skeletal tropomyosin; B, SS2; C, LS2; D, Nbs_2–LS2; E, LS2–Nbs_2 reaction quenched at time indicated by arrow in Figure 1; F, Nbs_2–SS2; G, SS2–Nbs_2 reaction quenched at time indicated by arrow in Figure 1. Amounts applied, 10 μg.

(Figure 2, slot D), indicating that at least one cross-link is present in all molecules. In order to determine the relationship between cross-link formation and the fast and slow phases of the kinetics, 0.5% SDS was added to quench the cross-linking reaction after most of the fast kinetic phase was completed (Figure 1), and the products were analyzed on gels. SDS unfolds and separates the chains rapidly, preventing further cross-linking. For both LS2 and SS2, products of the fast phase were un-cross-linked (Figure 2, slots E and G, respectively), indicating that cross-linking occurs during the slow phase.

It should be noted that the rate constants of the slow reaction for both LS2 and SS2 are close to that obtained for tropomyosin (Table I), a system for which the reaction with Nbs_2 produces complete cross-linking at Cys-190 (Lehrer, 1975). All of the SH groups in LS2 and SS2 are quite inaccessible as judged from the much smaller rate constants than that obtained in the case of 2-mercaptoethanol under similar conditions (Table I).

Tryptic Digestion of LS2 and SS2. Digestion of LS2 with trypsin has been shown to produce SS2 (Sutoh et al., 1978). The effects of disulfide cross-links on the course of trypsin digestion of LS2 were investigated by performing parallel reactions for 30 min at 25 °C on reduced LS2 and disulfide-cross-linked LS2 at various tyrpsin/LS2 ratios. The products formed were investigated by SDS–PAGE. Under conditions where digestion of reduced LS2 resulted in partial cleavage to SS2 (trypsin/LS2 = 0.02), digestion of cross-linked LS2 resulted in almost complete conversion to a pair of products (T_{1a} and T_{1b}) with mobilities approximately corresponding to M_r 100K and 90K (Figure 3). Since the number and location of cross-links can change the mobility and apparent molecular weight of peptides (Griffith, 1972), the mobilities of T_{1a} and T_{1b} were compared after reduction to obtain their chain weight. The procedure involved cutting out

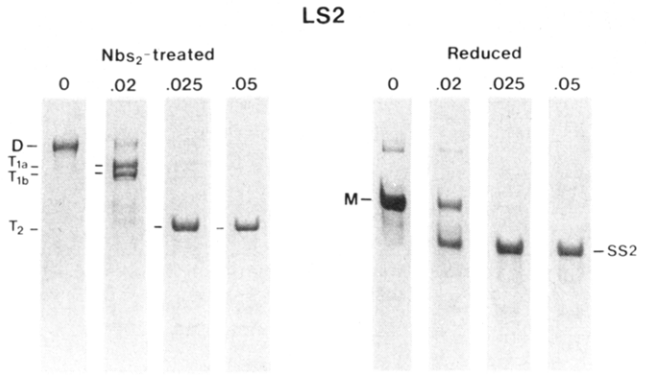

FIGURE 3: SDS gels of peptides produced by tryptic digestion of Nbs_2–LS2 (left) and reduced LS2 (right). Lanes are labeled with the weight ratio of trypsin/S2 reacted for 30 min at 21 °C in 0.05 M NaCl and 10 mM Hepes, pH 7.5. [LS2] = 2 mg/mL. M = monomer chains; D = dimer chains. amounts applied, 5 μg.

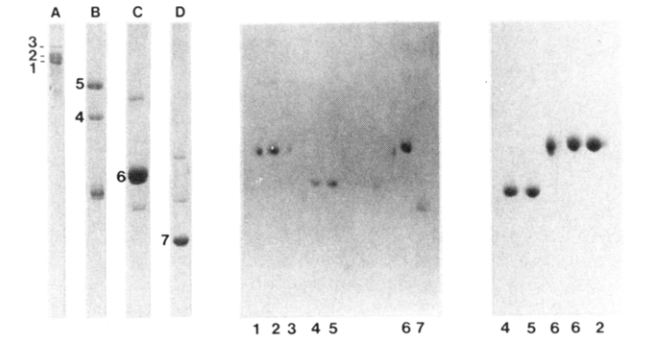

FIGURE 4: SDS gels of Nbs_2–LS2 and its tryptic peptides, T_{1a} and T_{1b}, before and after reduction. Left panel, before reduction: A, Nbs_2–LS2; B, products of mild trypsin treatment of Nbs_2–LS2, 5 = T_{1a}, 4 = T_{1b}; C, LS2; D, SS2. Middle and right panel, after reduction: numbered lanes in the middle and right panels correspond to the numbered bands in the left panel which were cut out, reduced, and rerun. Middle and right panels were obtained from separate gel runs. Note the following mobility relationships of the reduced peptides: 1 = 2 = 3 = 6 = LS2; 4 = 5; 7 = SS2.

Table II: Sequence Results of Edman Degradation of T_{1a} + T_{1b}

cycle	amino acid (nmol)
1	Leu (15.3), Lys (2.5)
2	Ala (8.5), Lys (2.3)
3	Glu (22.0), Lys (1.0)
4	Ala (7.6), Thr[a] (3.0)
5	Glu (22.8)
6	Thr[a] (2.7), Lys (1.3)

[a] Thr recovered as α-aminobutyric acid.

Table III: Sequence Results of Edman Degradation of T_2

cycle	amino acid (nmol)	cpm × 10^4
1	Lys (3.5)	7.2
2	Leu (6.7)	3.2
3	Glu (7.7)	2.2
4	Asp (3.7)	1.9
5	Glu (6.8)	3.9
6	Cys[a] (3.3)	28.5

[a] Cys recovered as alanine.

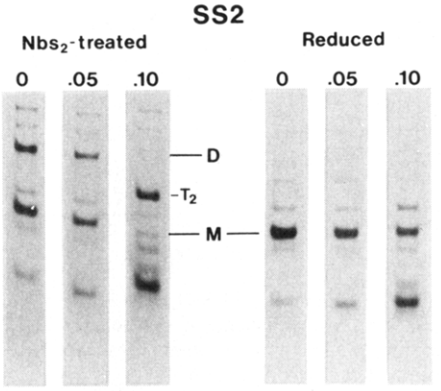

FIGURE 5: SDS gels of peptides produced by tryptic digestion of Nbs_2–SS2 (left) and reduced SS2 (right). Lanes are labeled with the weight ratio of trypsin/S2 reacted for 30 min; [SS2] = 2 mg/mL. For reaction conditions see Figure 3 legend. Amounts applied, 5 μg for lanes labeled 0 and .05; 9 μg for lanes labeled .10.

the stained bands, treating with dithiothreitol, and rerunning the reduced peptides on a reducing gel a second time (S. Wong and S. S. Lehrer, unpublished results). It was found that when the reduced T_{1a} and T_{1b} bands were rerun in adjacent slots in this manner, both peptides ran identically, each giving a single band corresponding to a chain weight of 50K (Figure 4, middle and right, slots 4 and 5). This suggests that T_{1a} and T_{1b} are disulfide cross-linked homodimers and the different mobilities observed are due to effects of differing number or location of disulfide cross-links. The introduction of disulfide cross-links in LS2 changes the initial cleavage position from one located about one-third from the carboxyl end of LS2 (~60 kDa) (which makes SS2, ~40 kDa) to a position located about one-sixth from one of the two ends (to make $T_{1a,1b}$ ~50 kDa).

To determine if the $T_{1a,1b}$ peptides are the product of cleavage at the N- or C-terminus, Edman degradation was performed on the mixture of T_{1a} and T_{1b} isolated by HPLC techniques. The results indicated the presence of two kinds of peptides, one with the original N-terminal sequence, Leu-Lys-(Lys or Ser)-Ala-Gln-Thr... (Table II), and the other that had two amino acids cleaved from the N-terminus, yielding (Lys or Ser)-Ala-Gln-Thr-Gln..., identical with that of SS2 (Lu, 1980). Thus, T_{1a} and T_{1b} are produced by trypsin cleavage of residues from the C-terminus of disulfide cross-linked LS2. From the estimated chain weight based on the mobilities of reduced LS2, SS2, T_{1a}, and T_{1b}, we concluded that the first trypsin cleavage point of cross-linked LS2 is near residue 360 ± 10. The actual cleavage site is most likely between residues 354 and 356 where the sequence is Arg-Lys-Lys (Lu & Wong, 1982, 1985).

At higher trypsin/LS2 ratios (0.025 or greater), T_{1a} and T_{1b} were cleaved to form a peptide (T_2) that had a mobility intermediate between reduced SS2 (40 kDa) and reduced LS2 (60 kDa) (Figure 3). A peptide of similar mobility was obtained by digestion of cross-linked SS2 with trypsin at higher ratios (Figure 5, trypsin/SS2 = 0.10) whereas reduced SS2 only partially degraded under those conditions (Figure 5). To determine if T_2 was disulfide cross-linked, to see if T_2 obtained from cross-linked LS2 was the same as T_2 obtained from cross-linked SS2, and to obtain its chain weight, the corresponding bands were cut out of the gels run in the absence of reducing agent and rerun in adjacent slots on reducing gels as described above. The results showed that T_2 obtained from both disulfide-cross-linked SS2 and LS2 had the same mobility corresponding to M_r 24K (Figure 6, slots 10 and 11). It should be noted, however, that T_2 from cross-linked LS2 was produced under lower trypsin concentration than T_2 from cross-linked SS2.

Further studies were performed to determine the cleavage site that led to T_2. T_2 obtained from disulfide-cross-linked LS2 was reduced and alkylated with iodo[^{14}C]acetate and subjected to Edman degradation yielding the N-terminal sequence Lys-Leu-Gln-Asp-Gln-Cys... (Table III). Comparison with

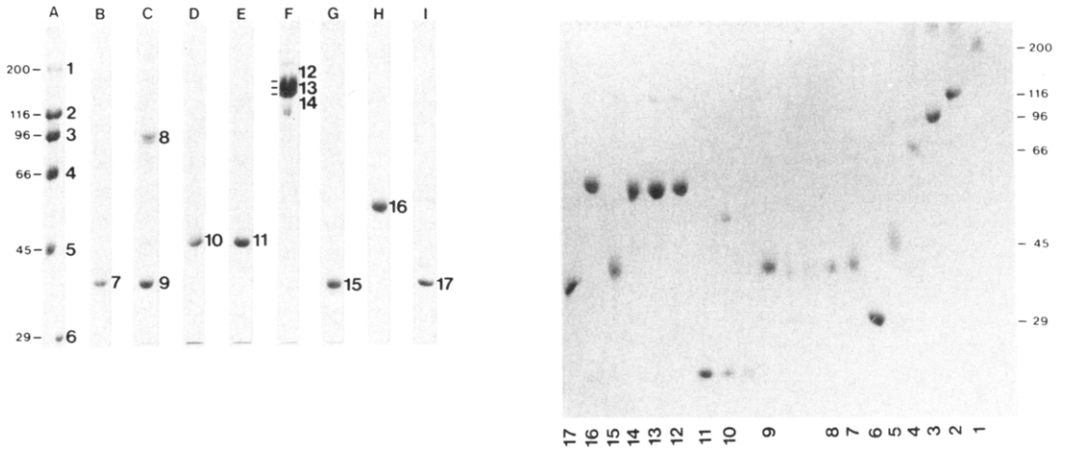

FIGURE 6: SDS gels of Nbs_2–LS2 and Nbs_2–SS2 and their tryptic peptide, T_2, before and after reduction. Left panel, before reduction: A, globular molecular weight markers in $M_r \times 10^{-3}$; B, SS2; C, Nbs_2–SS2; D, T_2 from SS2; E, T_2 from LS2; F, Nbs_2–LS2; G, SS2; H, LS2; I, rabbit skeletal tropomyosin. Right panel, after reduction: numbered lanes correspond to bands in left panel which were cut out, reduced, and rerun. Note the following mobility relationships of the reduced peptides in the right panel: 10 = 11 = reduced T_2; 7 = 8 = 9 = 15 = SS2; 12 = 13 = 14 = 16 = LS2.

the sequence of SS2 (Capony & Elzinga, 1981) shows that cleavage occurs at six residues before Cys-108, at the C-terminal side of Arg-102. Under the assumption that the C-terminus remained intact when SS2 was degraded to T_2, T_2 would contain residues 103–287. The calculated chain weight is $185 \times 115 = 21.3k$, where 115 is the mean residue weight, in reasonable agreement with the estimate of 24K based on mobility on SDS–PAGE, taking into consideration that molecular weights of myosin rod fragments estimated on the basis of mobility on SDS–PAGE when globular proteins were used as molecular weight standards are usually 15–20% higher than those determined by sedimentation equilibrium measurements (Sutoh et al., 1978) or sequence analyses. Since the same peptide, T_2, on the basis of mobility on gels, is produced by trypsin digestion of cross-linked LS2 and SS2, cleavage must also occur at the C-terminal portion of T_1, presumably at residue 287, when it is further degraded to T_2.

Thermal Unfolding Profiles of Reduced and Disulfide-Cross-Linked LS2. The thermal unfolding profiles were obtained by monitoring the ellipticity at 222 nm with increasing temperature (Figure 7). At the lowest temperature of measurement (7.5 °C) reduced and cross-linked samples at identical concentrations had the same ellipticity. The reduced LS2 shows major transition at about 45 °C in agreement with earlier studies (Sutoh et al., 1978). The major transition of the disulfide-cross-linked LS2 is shifted to about 52 °C, indicating a stabilizing effect of the cross-links. A pretransition is noted at physiological temperatures for cross-linked LS2 which is either not present or is quite small in the reduced LS2. This pretransition is associated with a greater degree of helix unfolding in the 25–42 °C temperature range for the disulfide-cross-linked LS2. Similar effects on helix unfolding due to a specific disulfide cross-link in tropomyosin have been observed (Lehrer, 1978).

Discussion

The ability to produce disulfde cross-links by reaction of both SS2 and LS2 with Nbs_2 shows that the two chains of the coiled-coil structures interact in register. Previous studies have shown that disulfide-cross-linked SS2 could be obtained by air oxidation of digested myofibrils (Ueno & Harrington, 1981a,b) and from Nbs_2 treatment of rods followed by trypsin cleavage (Stewart, 1982).

The schematic diagram (Figure 8) illustrates various kinds of fragments produced during the progress of tryptic digestion

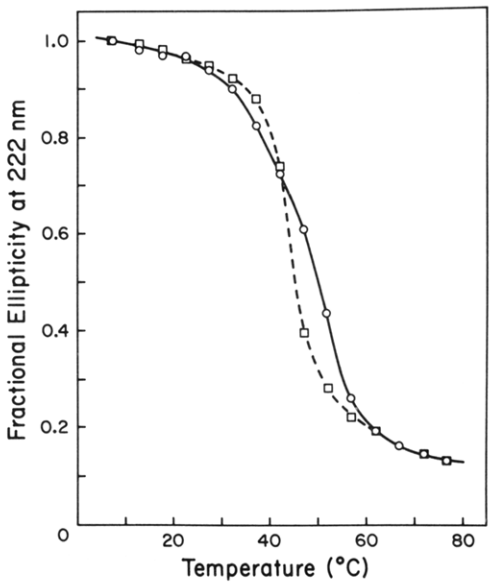

FIGURE 7: Thermal unfolding of reduced LS2 (□) and Nbs_2–LS2 (○). Conditions: 0.26 mg/mL protein solutions containing 0.6 M NaCl, 5 mM sodium phosphate buffer, pH 7.3, and 0.1 mM EDTA; 5 mM DTT included for reduced LS2.

and the most probable location of the cross-links. The product of Nbs_2 reaction with LS2 comprises >95% cross-linked species, revealed on gels chiefly as two closely spaced bands. SDS–PAGE indicated that all the cross-linked molecules remained cross-linked when T_2 and its precursor T_{1a} and T_{1b} were formed by tryptic digestion. Since T_2 contains only one pair of Cys at residue 108, LS2 must have been completely cross-linked at Cys-108 (Figure 8, top). The anomalously fast mobility of T_{1b} can be explained by the presence of an additional disulfide bond at Cys-66. The observation that the difference in mobility between two cross-linked products in the uncleaved cross-linked LS2 is much less than that between T_{1a} and T_{1b} suggests that the Cys-410 pair is cross-linked in both species, since, for a molecule that is already cross-linked at two well-separated regions in its primary structure, Cys-108 and Cys-410, the presence of a third cross-link at Cys-66 would not be expected to greatly increase its mobility. These interpretations were consistent with the kinetic results, indicating that somewhat more than two pairs reacted slowly with cross-linking.

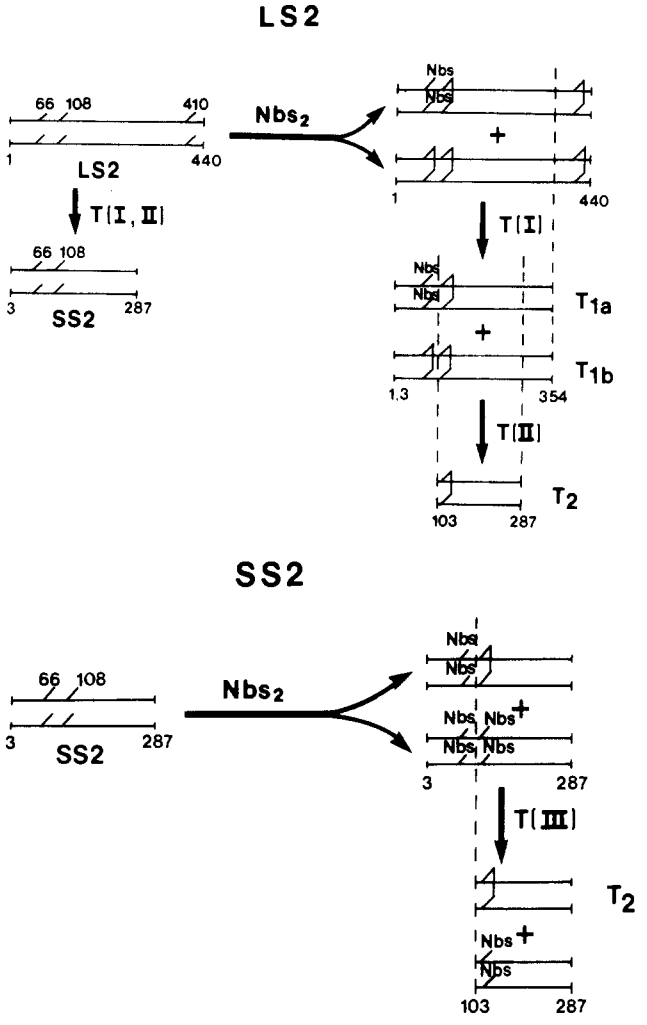

FIGURE 8: Location of disulfide bonds and pattern of tryptic (T) cleavage of reduced and Nbs_2-treated SS2 and LS2. Trypsin/substrate weight ratio indicated by T(I) = 0.02, T(II) = 0.025–0.5, and T(III) = 0.1. Cleavage points indicated by dashed lines.

Nbs_2 treatment of SS2 results in one cross-linked dimer species and un-cross-linked monomer as shown by SDS–PAGE, suggesting that only some molecules form a cross-link at one pair of Cys (Figure 8, bottom). Since all cross-linked molecules remained cross-linked when they were degraded to T_2, we conclude that Cys-108 must be the principal cross-link forming group whereas Cys-66 is the group that reacts without cross-linking. This is also consistent with the kinetics that indicated that more than half of the Cys of SS2 reacted during the fast phase without cross-linking.

The factors that determine whether two adjacent cysteines react with Nbs_2 to result in a disulfide link rather than Nbs-blocked species can be discussed in terms of information available for tropomyosin which quantitatively forms disulfide bonds between Cys-190 residues upon treatment with Nbs_2 (Lehrer, 1975). Studies with tropomyosin have suggested that the cross-linking of Cys cannot occur in the fully helical chain-closed state, since the S atoms of Cys-190's which are in coiled-coil a-type positions (McLachlan & Stewart, 1975) are about 7 Å apart, separated by the helical backbone. The cross-linking was therefore proposed to take place in a locally unfolded chain-open state (Lehrer et al., 1980). Examination of the recently determined sequence of LS2 (Lu & Wong, 1985) and SS2 (Capony & Elzinga, 1981) shows that all cysteines are in a-type positions. In the chain-open state, cross-linking between adjacent SH groups can occur by sulfhydryl–disulfide exchange after one SH reacts to form an Nbs-blocked intermediate, /–SSR HS–/ (where R = Nbs and the slant is the peptide backbone), if the first-order rate of internal exchange of RSS– with the neighboring free SH group to yield /–SS–/ is faster than the bimolecular rate of reaction of the free SH group with external Nbs_2 to yield /–SSR RSS–/. The observation in this work that reaction without cross-linking takes place during the fast phase of the biphasic reaction indicates that the cross-linking rate is much slower than the bimolecular rate with Nbs_2. Also, the observation that only a fraction of the molecules become cross-linked during the slow phase for SS2 indicates that the cross-linking rate is of the same order of magnitude as the bimolecular rate. Further studies are necessary to relate these rates with the reactivity of Cys in the two postulated states, chain closed and chain open, and the rates of fluctuation between these states. That two different rates were observed for the three reacting Cys, although they are all located at "a" positions, suggests that the rate is influenced by the nature of neighboring side chains and/or local conformation of the polypeptide chains. The observation that the same Cys behaves differently in LS2 and SS2, i.e., partial and no cross-linking at Cys-66 in LS2 and SS2, respectively, and complete and partial cross-linking at Cys-108 in LS2 and SS2, respectively, suggests that the extra stretch of polypeptide in LS2 at the C-terminus affects the conformation of the N-terminal portion of the molecule.

It is clear that the presence of disulfide cross-links in SS2 and LS2 changes the location of the trypsin cleavage points. For cross-linked LS2 cleavage takes place near residue 360 under conditions that un-cross-linked LS2 partially degrades to SS2 by cleavage at Arg-287. This appears to be due to the presence of the cross-link at Cys-410, 50 residues away. It is also possible that the disulfide bond at Cys-108 has some role in shifting the cleavage position from residue 287 to ~360. Furthermore, the disulfide bond at Cys-108 creates a new susceptible site, Arg-102, six residues away. If trypsin cleaves peptide bonds most readily in regions of the coil where the helix is locally unfolded, these results would suggest that disulfide cross-links not only can increase local unfolding but also can change the relative degree of unfolding in different regions of the coiled-coil structure; i.e., these effects can be transmitted at a considerable distance from the cross-link. It should be noted that early studies on digestion of aged HMM indicated that the degradation of a 37K fragment (presumably SS2) into a 25K fragment (presumably T_2) was inhibited by prior treatment with mercaptoethanol, suggesting that the presence of disulfide bonds affected the digestion patterns (Balint et al., 1975). Also, Reisler et al. (1983) have reported a new chymotryptic cleavage site in oxidized rod; further studies will be necessary to determine the relation of the trypsin cleavage site at Arg-102 with their chymotryptic cleavage site.

Previous work with tropomyosin has also shown that a disulfide bond at Cys-190 increases the unfolding in the physiological temperature range perhaps due to steric strain (Lehrer, 1978). Since the current studies show a similar destablization, such effects may occur in general in coiled-coil structures. Although disulfide bonds are found in purified preparations of tropomyosin and myosin fragments if reducing agent is omitted during preparation, it is not known if they exist in vivo or if the oxidation state of Cys is relevant to their mode of action. These results do in general suggest, however, that strain, produced perhaps at another part of the molecule, can produce significant conformational perturbations at some distance from the source, which may play an important role in the mechanism of contraction and regulation. In these cases, the perturbation may be the result of reversible interaction

with neighboring molecules or other protein subunits in filamentous structures.

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Registry No. Nbs_2, 69-78-3; Cys, 52-90-4; trypsin, 9002-07-7.

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