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Local Structural Changes in Tropomyosin Detected by a Trypsin-Probe Method†

Hitoshi Ueno

ABSTRACT: Structural changes in tropomyosin from rabbit skeletal muscle were studied by the tryptic digestion method, which is an application of the quantitative enzyme-probe method recently developed by Ueno and Harrington [Ueno, H., & Harrington, W. F. (1984) *J. Mol. Biol. 173*, 35–61]. Effects of ionic strength, temperature, and an interchain disulfide bond at Cys-190 on the structure of tropomyosin were examined. A region of high susceptibility to trypsin was found to be localized in the middle portion of the molecule, and its susceptibility increased on lowering ionic strength and/or raising temperature. With the introduction of a disulfide bond at Cys-190, cleavage on the N-terminal side of Cys-190 was accelerated. The results suggest that skeletal muscle tropomyosin is flexible in the middle of the molecule in contrast to the flanking N- and C-terminal trypsin-resistant segments.

In a recent study (Ueno & Harrington, 1984), proteolytic enzymes were used to quantitatively probe local conformational changes within the α-helical region of the myosin rod. In this enzyme-probe method, the kinetic analysis of proteolytic digestion and the sites of cleavage in the myosin rod were determined by following the time course of fragmentation on SDS[1]-containing gels. Since the method can be applied to any other system as well and is particularly powerful to detect local conformational changes, it was extended in this study to probe local conformational changes in tropomyosin.

Tropomyosin plays an important role in the regulation of vertebrate skeletal muscle together with troponin (Ebashi & Endo, 1968), as well as in various cell motile systems. Rabbit skeletal muscle tropomyosin is an α-helical coiled-coil molecule composed of 284 amino acid residues per polypeptide chain (Stone & Smillie, 1978; Sodek et al., 1978). Although an exact regulatory mechanism of this protein is still in controversy, it is likely that some conformational changes in tropo-

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[1] Abbreviations: TM, rabbit skeletal α-tropomyosin; TM_{SH}^{SH} or reduced TM, TM without an interchain disulfide bond; TM_{S}^{S} or oxidized TM, TM with an interchain disulfide bond at Cys-190; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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myosin play an important role on the thin filament in situ. In fact, a local conformational transition in tropomyosin has been suggested as a distinct possibility to be involved in the regulatory mechanism (Lehrer, 1978; Lehrer et al., 1981; Potekhin & Privalov, 1982). Therefore, it is interesting to identify the location of the conformational change in tropomyosin if any. Since trypsin has been extensively used to probe tropomyosin structure qualitatively (Ooi, 1967; Eckard & Cowgill, 1976; Ueno & Ooi, 1978; Gorecka & Drabikowski, 1977; Pato & Smillie, 1978), this enzyme was again used in this study but to quantitate the kinetics of the cleavage process. Introduction of a disulfide bond at Cys-190 and changes in ionic strength and temperature were employed as perturbants of the local tropomyosin conformation. The results obtained by the present enzyme-probe technique are compared with those obtained from various physicochemical studies.

Materials and Methods

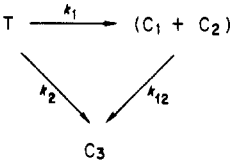
Proteins. Tropomyosin was obtained from rabbit psoas skeletal muscle according to the method of Bailey (1948). α-Tropomyosin fractionated on hydroxylapatite column (Eisenberg & Kielley, 1974) was exhaustively dialyzed vs. 20 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5), and the resulting paracrystals were collected by centrifugation (5000 rpm, 5 min) to remove low molecular weight peptides, if any. The final yield of α-tropomyosin (TM) was 97%, which was determined by a densitometric tracing of a gel. The oxidized form of TM (TM_S^S) was prepared by the procedure of Lehrer (1975), and its yield of thiol oxidation employed in this study was 98%. TM solutions of 1.0 mg/mL were prepared by exhaustive dialysis against several changes of appropriate solutions for 1 to 2 days (TM, $E^{0.1\%}_{278nm}$ = 0.33 cm^2 mg, molecular mass 66000 g/mol). Trypsin and trypsin inhibitor were purchased from Worthington Biochemical (Freehold, NJ). Trypsin was dissolved in 1 mM HCl until use.

Enzyme Assays. Intrinsic enzyme activity of trypsin (k_{int}) was determined by the method of Hummel (1959) with modifications as follows. Effect of ionic strength on trypsin was examined at 24 °C with a solution containing trypsin (2.0 μg/mL), 0.37 mg/mL (1.0 mM) *p*-toluenesulfonyl-L-arginine methyl ester (TAME), and 20 mM cacodylic acid. Ionic strength (μ) was adjusted to 0.015–1 M with NaCl and pH readjusted to 7.0 with HCl. Hydrolysis rate was determined from ΔA_{247} per minute by a temperature-controlled (±0.5 °C) Perkin-Elmer spectrophotometer between A_{247} = 0 and A_{247} = 0.20 (ΔA_{247}/min = 0.54 corresponds to 1 unit). The relative rates thus determined are 100 (μ = 0.15 M), 92 (μ = 0.025 M), 77 (μ = 0.055 M), 65 (μ = 0.115 M), 48 (μ = 0.42 M), and 40 (μ = 1.0 M). The effect of temperature on trypsin was examined with a solution containing trypsin (3.3 μg/mL), 1 mM TAME, 20 mM cacodylate (pH 7.9), and 0.15 M NaCl. ΔA_{247}/min values were 0.012 (5 °C), 0.026 (10 °C), 0.044 (15 °C), 0.067 (20 °C), 0.113 (25 °C), 0.173 (30 °C), 0.261 (35 °C), 0.360 (40 °C), 0.500 (45 °C), 0.645 (50 °C), and 0.84 (55 °C). β-Mercaptoethanol at 1–10 mM had little affect on the hydrolysis rate.

Tryptic Digestion of Tropomyosin. A 10 μM TM solution (0.66 mg/mL) was used unless otherwise stated for digestion studies. Digestion was quenched by adding 0.1 volume of 0.4 mg/mL trypsin inhibitor. An equal volume of solution consisting of 3% SDS, 30% glycerol, 0.15 M Tris-HCl (pH 6.8), and a trace of bromphenol blue was added to the digested samples, and they were immediately heated. β-Mercaptoethanol at 50 mM was added when necessary.

Sodium Dodecyl Sulfate Containing Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out according to the method of either Laemmli (1970) or Weber & Osborn (1969) using slab gels. Acrylamide to methylenebis(acrylamide) was always 30/0.80 (w/w), and gel concentrations are given in acrylamide concentration (%) in the text. Peptide bands were stained in 0.2% Fast Green FCF, 7% acetic acid, and 50% methanol for about 12 h. Destaining was first done in 7% acetic acid and 50% methanol for about 2 h followed by several soakings in 7% acetic acid and 10% methanol. Peptide band intensities were determined by densitometric tracing of gels with a gel scanner (Helena Laboratories, Beaumont, TX) and a 610-nm filter (major absorbance range 350–550 nm). Maximum loading of proteins on gels was ~10 μg/band in order to use the linear range of absorbance of the spectrophotometer. All bands were assumed to have the same staining coefficient. Peptide band intensity was reduced to a relative molar ratio by using the molecular weight of the peptide species estimated from its electrophoretic mobility in SDS gels according to Weber & Osborn (1969). Molecular weight standards used to estimate M_r values of the tryptic fragments are as follows: intact tropomyosin (M_r 32700; Stone & Smillie, 1978), CNBr fragments of TM (M_r 13500 for Cn1A and M_r 16000 for Cn1B; Hodges & Smillie, 1973), and cyanylation fragments of TM (M_r 21700 and 10900) prepared by the method of Ueno et al. (1977).

Kinetic Analysis. The time course of fragmentation of tropomyosin by tryptic cleavage was analyzed on the basis of the following scheme (Ueno & Harrington, 1984):



where T and C_i represent intact tropomyosin and its C-terminal fragment of TM, respectively (see details in text). k_1, k_2, and k_{12} are first-order rate constants. Then, $-d[T]/dt = (k_1 + k_2)[T]$, $d[C_1 + C_2]/dt = k_1[T] - k_{12}[C_1 + C_2]$ and $d[C_3]/dt = k_2[T] + k_{12}[C_1 + C_2]$. Solutions of these equations were given previously (Ueno & Harrington, 1984). Since concentrations $[C_1]$ and $[C_2]$ could not be easily determined from experiments particularly at the early stage of the cleavage, $[C_1 + C_2]$ was calculated from the equation $[C_1 + C_2] = [T]_0 - ([T] + [C_3])$, where $[T]_0$ is the molar concentration of TM at $t = 0$. The validity of this equation was occasionally tested by experiments as follows. The C-terminal fragments in the oxidized form (with a disulfide bond) were separated on the SDS gels in the absence of β-mercaptoethanol from the N-terminal fragments and quantitated unambiguously [see Figure 2 (1) for examples]. It was found that calculated values were always close to the measured ones (±10%). Appropriate rate constants, k_i, were tested by trail and error to fit the data with a Techtronix (4051) computer. Figure 3 gives examples. [T] and $[C_3]$ were experimentally determined. The cleavage decay rate of TM, k_0, is given by $k_0 = k_1 + k_2$.

Results

Tryptic Cleavage of Tropomyosin. Two preparations of tropomyosin were used, reduced and disulfide cross-linked. Tropomyosin can be intramolecularly cross-linked because the two polypeptide chains of skeletal muscle tropomyosin are in register; the interchain disulfide bond at Cys-190, which is the only cysteine residue in the sequence, was introduced into the molecule by reaction with 5,5′-dithiobis(2-nitrobenzoate) (Lehrer, 1975). The two forms of tropomyosin with and without an interchain disulfide bond thus prepared were di-

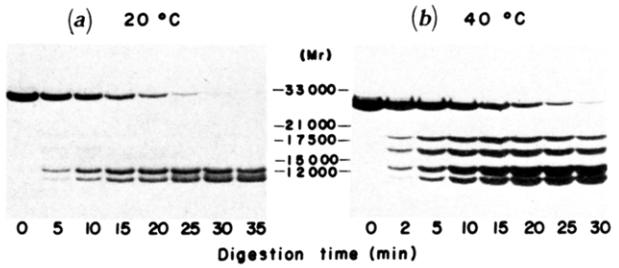


FIGURE 1: SDS-containing gel electrophoresis of tropomyosin digested by trypsin at 20 (panel a) and 40 °C (panel b). Digestion conditions: 0.67 mg/mL (10 μM) reduced TM (TM^{SH}_{SH}), trypsin at 2.0 μg/mL at 20 °C and at 0.10 μg/mL at 40 °C, 20 mM cacodylic acid, 1 mM EDTA, 0.25 M NaCl, and 1 mM β-mercaptoethanol (pH 7.05 ± 0.02 at 25 °C).

gested by trypsin, and aliquots of the digestion mixture were removed at various reaction times. Proteolysis was quenched by addition of trypsin inhibitor, and the whole digest run on SDS-containing gels as shown in Figure 1. The absorbance for each peptide band was determined by a densitometer and reduced to relative molar intensity, I_r, by correcting for its corresponding M_r value. The time course of the decay of the tropomyosin band could be followed by plotting log (undigested tropomyosin intensity) vs. time, which gave a pseudo-first-order rate constant.

The effect of the enzyme to substrate ratio on the cleavage rate for tropomyosin was examined. When initial substrate (tropomyosin) concentrations (0.50 mg/mL) were fixed and enzyme (trypsin) concentrations varied (2–10 μg/mL), the cleavage rate constant for tropomyosin (k) was proportional to enzyme concentration. On the other hand, the rate constant (k) was independent of the initial substrate concentrations ([S]) (0.1–1.0 mg/mL) when the enzyme concentration was fixed at 2.0 μg/mL. These observations are expected if the cleavage reaction can be treated with the formulation of the Michaelis–Menten kinetics when [S(tropomyosin)] $\ll K_m$ [see Ueno & Harrington (1984)]. Under all the experimental conditions used in the present study, log (undigested tropomyosin intensity) vs. digestion time plots were linear. Therefore, the kinetics of fragmentation was analyzed by using first-order rate constants in this study.

Tryptic fragmentation patterns of tropomyosin on SDS gels were very similar at constant temperature among various enzyme to substrate ratios used and essentially *independent* of the [E]/[S] ratio (data not shown). The cleavage rates were *proportional* to trypsin concentration. However, the cleavage patterns were affected by the conditions when other conditions (e.g., temperature) were varied. Figure 1 shows examples of the digestion at 20 and 40 °C. The fragmentation patterns were clearly different between the two experiments and *dependent* on temperature. It should be noted again that the cleavage patterns were independent of the [E]/[S] ratio, but the cleavage rates were proportional to trypsin concentration at a fixed temperature as was shown previously (Ueno & Harrington, 1984). Therefore, it seems clear that significant changes in the local structure of tropomyosin induced by changes in experimental conditions such as temperature will be detected by the trypsin-probe method.

Assignments of the Tryptic Cleavage Sites. Since the position or region where conformational changes are taking place will be examined in the present study, tryptic cleavage sites in tropomyosin had to be assigned unambiguously. Under the present experimental conditions, five major polypeptide bands were observed on SDS gels (in the presence of β-mercaptoethanol) for the tryptic digests of tropomyosin. The

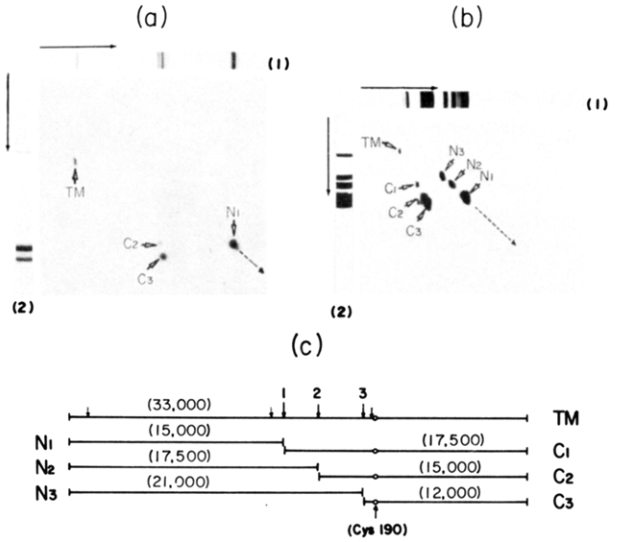


FIGURE 2: Two-dimensional SDS gel electrophoresis of oxidized tropomyosin digested by trypsin. Gel electrophoresis was performed according to Laemmli (1970) with 7% stacking and 14% separation gels. Digested tropomyosin was run on the gels in the absence of β-mercaptoethanol for the first-dimension gels (left to right). SDS gel patterns used for the first dimension are shown on top of the panels as indicated by (1). Gels were then sliced and soaked in the running buffer solution plus 0.1 M β-mercaptoethanol for 30 min. These reduced gels were then applied onto 14% separation gels without a stacking gel for the second dimension (top to bottom). SDS gel patterns of the digested TM samples reduced with β-mercaptoethanol before electrophoresis are shown on the left sides of the panels as indicated by (2) to compare the position of peptide bands. Digestion conditions: (panel a) 24 °C, 1.5 μg/mL trypsin for 10 min; (panel b) 40 °C, 0.10 μg/mL trypsin for 25 min; 20 mM cacodylate and 0.10 M NaCl (pH 7.0). Dashed lines indicate directions of diagonal spots, N_1 in (a) and N_1–N_3 in (b). C_i and N_i are described in panel c. (Panel c) Schematic representation of tropomyosin and its tryptic peptides. Locations of tryptic peptides are illustrated. Major cleavage sites (1–3) are shown by the arrows and minor ones by the broken arrows. Molecular weights (per polypeptide chain) are shown in parentheses.

bands are M_r 33 000 (undigested tropomyosin), 21 000, 17 500, 15 000, and 12 000 bands (see Figure 1). The relative intensities of those bands were dependent on experimental conditions used for the digestion. For example, M_r 21 000 and 17 500 bands were much more distinct at 40 than at 20 °C (Figure 1). Location of these peptides in the original amino acid sequence by SDS gel electrophoresis was based on two criteria: (1) C-terminal peptides including Cys-190 can be identified by a thiol-oxidation method, and (2) various tryptic peptides have already been identified by Pato & Smillie (1978).

Oxidized tropomyosin with an interchain disulfide bond at Cys-190 (TM^{S}_{S}) was digested by trypsin at 24 and 40 °C and run on SDS gels in the absence of β-mercaptoethanol (see Figure 2). The gels were then sliced and applied onto the second gels after reducing the disulfide bond as described in the legend of Figure 2. N-Terminal fragments are expected to migrate at diagonal positions in the two-dimensional gels while C-terminal ones migrate at off-diagonal positions. Three distinctive N-terminal fragment spots and three C-terminal spots were observed for the tryptic digestion at 40 °C (Figure 2, panel b). Molecular weights estimated for each fragment spot were as follows (peptides of Figure 2 are in parentheses): 21 000 (N_3), 17 500 (N_2), 15 000 (N_1), 17 500 (C_1), 15 000 (C_2), and 12 000 (C_3). N_3, N_2, C_1, and C_2 spots for the digestion at 20 °C (Figure 2, panel a) were very weak. The M_r 17 500 band (N_2 + C_1) and M_r 15 000 band (N_1 + C_2) were mixtures of the N- and C-terminal fragments, which were not resolved on SDS gels in the presence of β-mercaptoethanol.

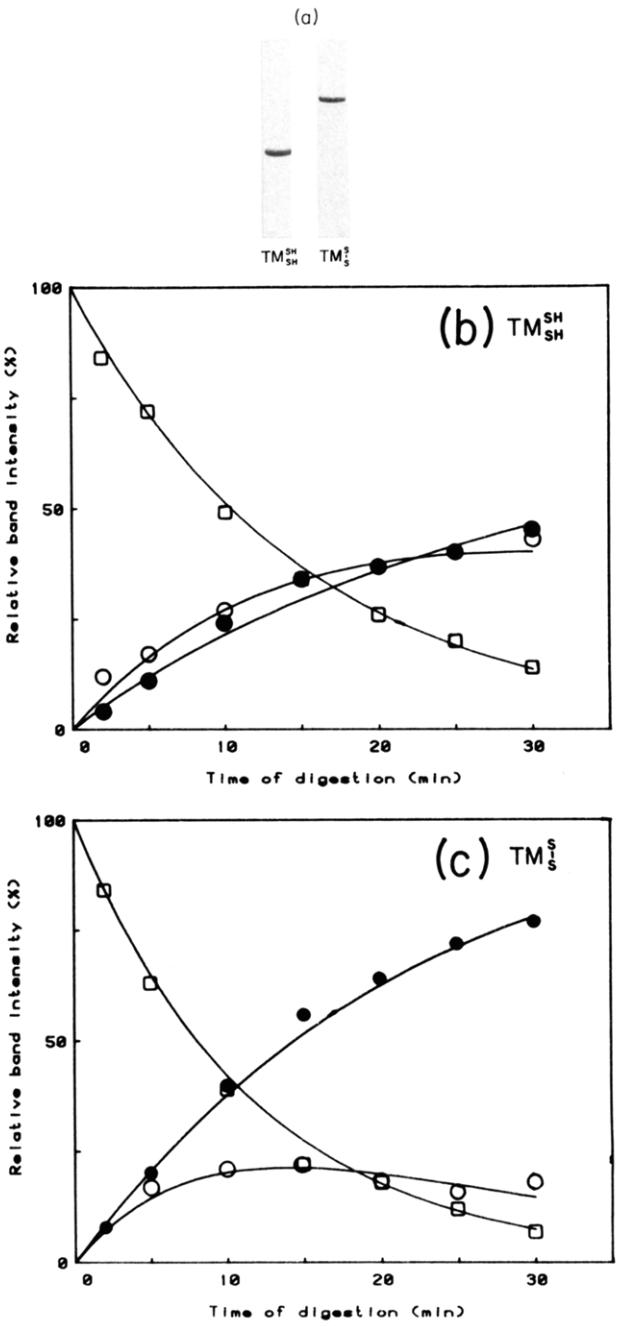
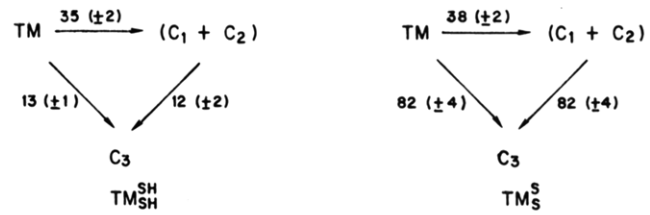


FIGURE 3: Kinetic analysis of tryptic digestion of tropomyosin. (Panel a) SDS-containing gel electrophoresis of tropomyosin used in this study. Fully reduced tropomyosin (TM^{SH}_{SH}) and oxidized tropomyosin (TM^{S}_{S}) are shown. Oxidation of TM is described under Materials and Methods. Gels of 14% were used according to the method of Laemmli (1970). Molecular weights are 32700 and 65300 for single and double chains of tropomyosin, respectively (Stone & Smillie, 1978). (Panels b and c) Tropomyosin (0.67 mg/mL) was digested by trypsin (0.50 μg/mL) in 20 mM cacodylate and 0.15 M NaCl (pH 7.0) at 30 °C. Peptide band intensities were reduced to molar intensities as described under Materials and Methods and plotted vs. digestion time. Digestion kinetics are analyzed on the basis of the scheme in the text. (□) Undigested TM, (○) $C_1 + C_2$, (●) C_3. Rate constants used for analysis: (panel b, reduced TM) $k_1 = 2.4\ h^{-1}$, $k_2 = 1.6\ h^{-1}$, and $k_{12} = 0.8\ h^{-1}$; (panel c, oxidized TM) $k_1 = 2.5\ h^{-1}$, $k_2 = 2.7\ h^{-1}$, and $k_{12} = 3.5\ h^{-1}$.

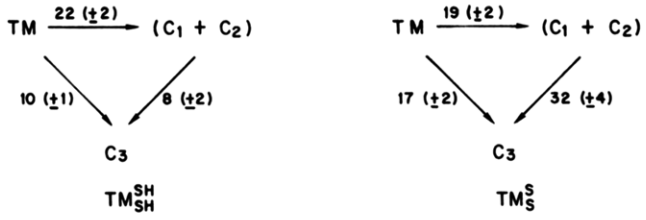
There seem to be three distinctive tryptic cleavage sites in the middle of the tropomyosin molecule that give rise to three C-terminal (C_1, C_2, C_3) and three N-terminal (N_1, N_2, N_3) fragments. Since the sum of molecular weights of C_i and N_i is very close to the molecular weight of tropomyosin (M_r 33000 on SDS gel), a cleavage at site i will produce N_i and C_i fragments. Two of the three cleavage sites have already been identified by Pato & Smillie (1978). Assignment of the tryptic fragments in this study was attempted by comparing them with those obtained by the method of Pato & Smillie (1978). Tryptic fragments of tropomyosin identified by Pato & Smillie (1978) are residues 1–133 (M_r 15300), residues 13–125 (M_r 13000), residues 134–284 (M_r 17400), residues 183–284 (M_r 11700), and residues 183–244 (M_r 7100). Two distinctive cleavage sites are therefore located near Arg-133–Ala-134 and Arg-182–Ala-183 peptide bonds. The most likely locations of the fragments are residues 134–284 for C_1 and residues 183–284 (and/or 179–284) for C_3, respectively. Another site that produces $N_2 + C_2$ fragments seems to be located somewhere between these two sites. It is worth noting that sizes of the tryptic fragments formed were the same (or similar) between the reduced and oxidized forms of tropomyosin. A summary of the assignment of these tryptic fragments is illustrated in Figure 2c. The N-terminal end region contains a high density of trypsin-sensitive sites (Lys-5–Lys-6–Lys-7 and Lys-12), but the cleavage at this region, if any, was not investigated in this study. Since the intensities of the C-terminal fragments could be determined much less ambiguously than those of the N-terminal ones, the C-terminal fragments as well as undigested tropomyosin were used for kinetic analysis in this study.

Kinetic Analysis of the Tryptic Digestion of Tropomyosin. The tryptic digestion mode of tropomyosin was analyzed on the basis of the kinetic scheme described under Materials and Methods. Examples of the analyses are shown in Figure 3. Either fully reduced tropomyosin (TM^{SH}_{SH}) or oxidized tropomyosin (TM^{S}_{S}) was digested by trypsin at 30 °C, pH 7.0 and $\mu = 0.15$ M. Undigested tropomyosin and the C-terminal fragments $C_1 + C_2$ and C_3 were quantitated as described above. Time course of the fragmentation mode was simulated by using three different rate constants (k_1, k_2, and k_{12}). Decay curves for undigested tropomyosin (squares in Figure 3) are uniquely functions of $k_1 + k_2$ in the simulation. The first-order rate constants of cleavage obtained from the analyses were normalized for the intrinsic hydrolysis rate of trypsin by using *p*-toluenesulfonyl-L-arginine methyl ester as a model substrate, and the normalized rate constants, k (h^{-1}/units), are compared in this study.

Effects of Ionic Strength on Tropomyosin. It has been known for some time that the stability of tropomyosin depends on ionic strength; i.e., it is more stable in high ionic strength solvents (Woods, 1969). It was therefore of interest to examine the location of the region(s) in tropomyosin that is most sensitive to ionic strength. Reduced and oxidized forms of tropomyosin were probed by trypsin as described above at 24 °C (see Figure 4). Reduced tropomyosin (TM^{SH}_{SH}) showed much less ionic strength dependence than oxidized tropomyosin (TM^{S}_{S}). A marked difference between the two forms was seen in the cleavage at site 3 (filled circles in Figure 4) close to Cys-190. TM^{SH}_{SH} showed virtually no effect of ionic strength at this site whereas TM^{S}_{S} was highly susceptible to trypsin at low ionic strength ($\mu < 0.1$). Kinetic analyses at $\mu = 0.015$ M were as follows (numbers are normalized rate constants, h^{-1}/units):

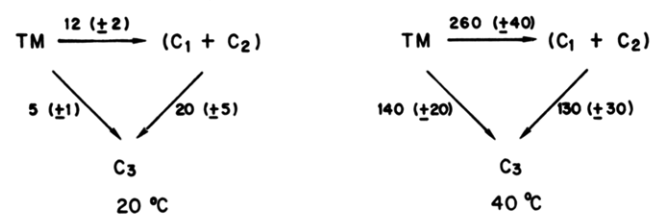


While the analyses at μ = 1.0 M were as follows:



Fractionation of Tryptic Fragments of Tropomyosin. Gorecka & Drabikowski (1977) first observed that the mode of tryptic fragmentation was affected by the interchain disulfide bond, which is consistent with the observations presented above. Since the tryptic fragments can be fractionated by QAE-Sephadex column chromatography (Ueno & Ooi, 1978), fractionation of the fragments was reexamined by taking the present observations into account. Tropomyosin was digested by trypsin at pH 7.5, μ = 0.20 M and 24 °C, and the digests were fractionated as shown in Figure 5. The N-terminal fragment (N_1 in this particular case) was eluted at lower NaCl concentration than the C-terminal fragments (C_3 and C_2 for TM^{SH}_{SH}, C_3 for TM^{S}_{S}). Since tyrosine residues are localized in the C-terminal half of the molecule (Stone & Smillie, 1978), the C-terminal fragments show higher absorbance than the N-terminal fragment. A distinct difference between reduced and oxidized tropomyosin in the digests is the presence of the C_2 (M_r 15 000) fragment for TM^{SH}_{SH}. This observation again supports the view that TM^{S}_{S} is more susceptible to trypsin near Cys-190 than TM^{SH}_{SH} and provides unambiguous identification of the various tryptic fragments.

Effect of Temperature on Tropomyosin. To investigate the temperature dependence of tropomyosin, digestions were performed at 5–40 °C, pH 7.0 and μ = 0.15 M. Fragmentation modes at various temperatures were qualitatively compared in two different ways (Figure 6), where tryptic digests of tropomyosin on SDS gels are shown. Panel a in Figure 6 shows a family of cleavage patterns where the intrinsic trypsin hydrolysis rate for a model compound (TAME) was kept constant, and panel b shows those where 60% of the tropomyosin band (M_r 33 000) is digested. It is clear from the comparison that tropomyosin was more susceptible to trypsin at higher temperature and that fragmentation patterns are temperature dependent. However sizes of the individual fragments remained the same within the resolving ability of the bands on SDS gels over the temperature range 5–40 °C. The M_r 11 000 fragment (no. 6 in Figure 6) was most likely residues 189–284 (containing Cys-190) and analyzed as a part of the C_3 fragment for convenience. Since the comparison of the cleavage patterns over the wide temperature range shows markedly different temperature dependencies among the various cleavage sites, the temperature dependence of these individual site rate constants was quantitatively analyzed on the basis of the same scheme used above. Examples of kinetic analyses for the reduced tropomyosin digested at 20 and 40 °C (see Figure 1) were as follows (numbers are normalized rate constants, h^{-1}/units):



For this situation, $k_2 < k_{12}$ at 20 °C, and $k_2 \approx k_{12}$ at 40 °C.

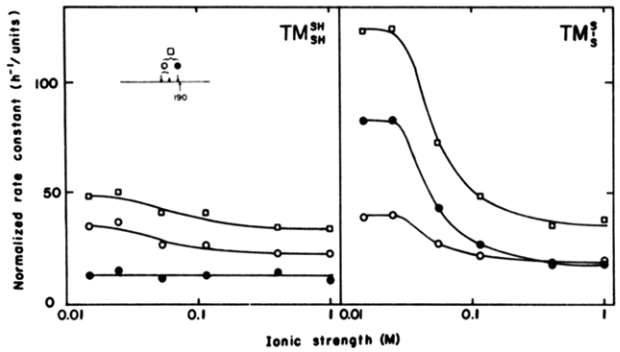


FIGURE 4: Effect of ionic strength on the tryptic digestion of tropomyosin. Cleavage rate constants (h^{-1}) obtained as in Figure 3 were normalized for intrinsic hydrolysis rates of trypsin (units). Normalized rate constants (h^{-1}/units) obtained at various ionic strengths are shown. Experimental conditions: 0.67 mg/mL tropomyosin, 20 mM cacodylate (pH 7.1), 24 °C, 1.5 μg/mL trypsin. Ionic strength was adjusted with NaCl. (Left) Reduced tropomyosin and (right) oxidized tropomyosin. (□) Total cleavage rate of TM, (○) cleavage rate at $C_1 + C_2$ sites, and (●) cleavage rate at C_3 site.

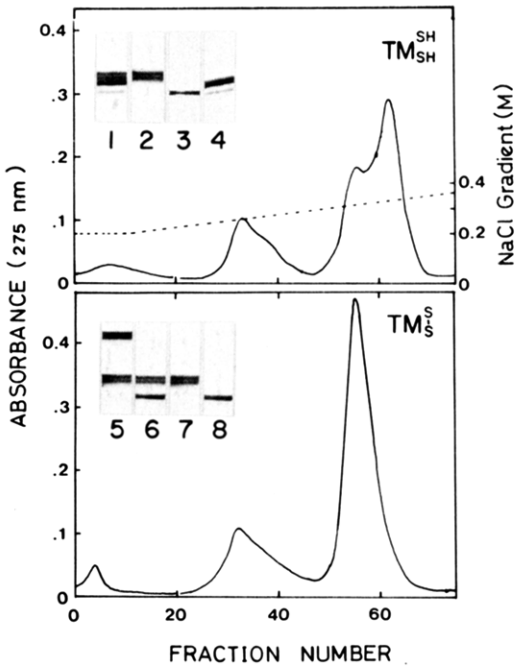


FIGURE 5: Fractionation of tryptic fragments of tropomyosin on a QAE-Sephadex A-50 column. A total of 100 mg of either oxidized tropomyosin (TM^{S}_{S}) or reduced tropomyosin (TM^{SH}_{SH}) was digested by trypsin (1.5 μg/mL) in 20 mM cacodylic acid, 0.2 M NaCl, and 1 mM EDTA (pH 7.1) at 24 °C for 30 min and the cleavage quenched by trypsin inhibitor. The cleavage products were exhaustively dialyzed vs. solution A (0.20 M NaCl and 20 mM Tris-HCl, pH 7.5) and then loaded on the QAE-Sephadex column (1.4 cm × 10 cm) equilibrated with solution A. A linear gradient from 0.20 M NaCl (solution A, 200 mL) to 0.40 M NaCl (200 mL) was used for elution, and 5 mL was collected per fraction. Elution profile was determined from the absorbance at 275 nm. (Top panel) Reduced TM; SDS gel patterns in the insert: (lane 1) total digests ($N_1 + C_2 + C_3$), (lane 2) fraction 33 (N_1), (lane 3) fraction 55 (C_3), and (lane 4) fraction 63 (C_2). (Bottom panel) Oxidized TM; SDS gel patterns in the insert: (lane 5) total digests (in the absence of β-mercaptoethanol), (lane 6) total digests (in the presence of β-mercaptoethanol, $N_1 + C_3$), (lane 7) fraction 33 (N_1), and (lane 8) fraction 55 (C_3). SDS gel electrophoresis was performed according to Laemmli (1970) with 15% gels.

The C_1 (and/or C_2) fragment was rapidly degraded into C_3 fragment at lower temperature, while $C_1 + C_2$ and C_3 fragments were produced by trypsin at a comparable rate at higher temperature. Therefore, the apparent yield of high molecular weight tryptic fragments (M_r 21 000 and 17 500) was high at 40 °C but low at 20 °C.

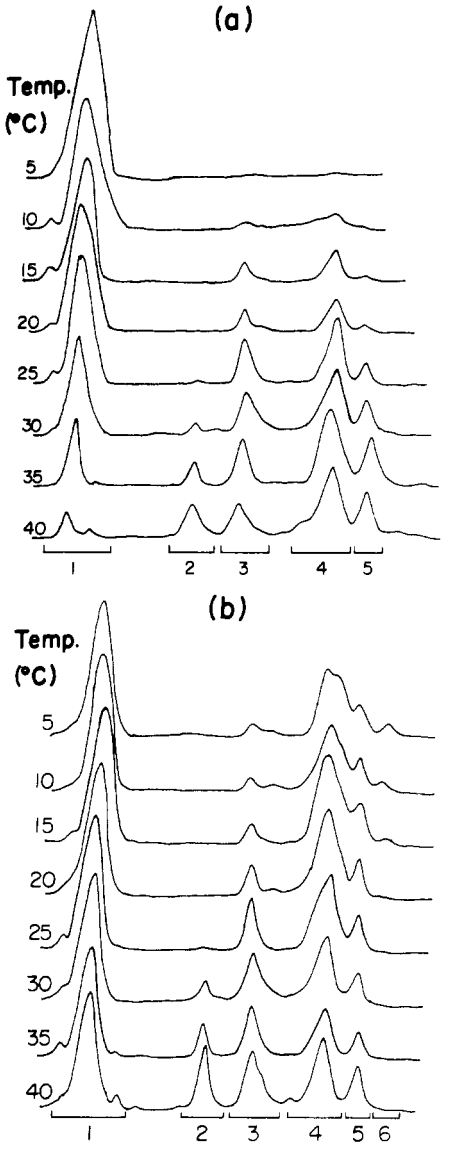


FIGURE 6: Comparison of SDS-gel patterns of tropomyosin digested by trypsin at various temperatures. Reduced tropomyosin (TM^{SH}_{SH}) was digested in 20 mM cacodylate, 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.15 M NaCl (pH 7.05). (a) Cleavage patterns are compared at a stage $xy = 0.36$, where x is trypsin intrinsic activity (in units) and y is time (in minutes). (b) Cleavage patterns with 60% TM digested are compared. Molecular weights of peptides (1–6 at bottom of the figure) estimated on SDS-gels are as follows: 1, 33 000; 2, 21 000 (N_3); 3, 17 500 ($N_2 + C_1$); 4, 15 000 ($N_1 + C_2$); 5, 12 000 (C_3); 6, 11 000. See also Figure 1 for the SDS gel electrophoresis.

The normalized cleavage rate constants increased with temperature in both reduced and oxidized tropomyosin (Figure 7). The cleavage rates for TM^{S}_{S} were always higher than those for TM^{SH}_{SH} under the present conditions. The rates for TM^{SH}_{SH} increased exponentially with temperature while those for TM^{S}_{S} showed distinctive transitions (Figure 7), which is clearly seen in derivative plots (dotted lines). Therefore, it appears that an introduction of the interchain disulfide bond into the tropomyosin molecule destabilizes the local region around residues 130–190 and the conformational transition in this region is clearly separated from the rest of the molecule. The apparent heats of activation can be obtained from Arrhenius plots (log k vs. $1/T$) of the normalized cleavage rate constants. The apparent heats of activation, estimated from each slope in the Arrhenius plots, are listed in Table I. $\Delta H^{\ddagger}$ for TM^{S}_{S} was 10–20% higher for primary cleavage (k_0, k_1, k_2) than for TM^{SH}_{SH}. A secondary cleavage (k_{12}) showed lower $\Delta H^{\ddagger}$ values

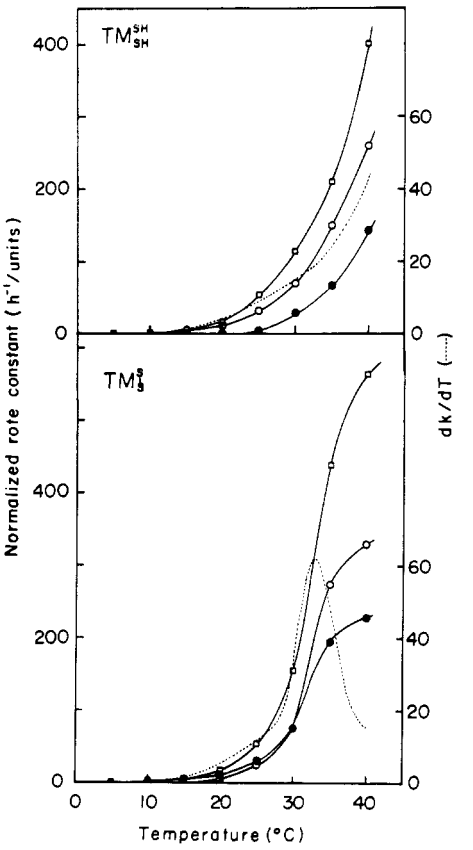


FIGURE 7: Temperature dependence of the trypsin cleavage rate constants of tropomyosin. TM digested by trypsin at pH 7.05 and $\mu = 0.15$ M was analyzed as in Figure 3, and the normalized rate constants are plotted vs. temperature. Symbols are the same as those in Figure 4. Data obtained from reduced (top) and oxidized (bottom) TMs are shown. Dotted lines, which are derivatives of the total cleavage rate (□), were obtained graphically.

Table I: Apparent Heat of Activation Obtained by Tryptic Digestion Method: A Summary of Figure 7

TM species	kinetic rate constants used for analysis of $\Delta H^{\ddagger}$ (kcal/mol)[a]			
	k_0	k_1	k_2	k_{12}
TM^{SH}_{SH}	29	28	28	16
TM^{S}_{S}	36	38	31	17

[a] See details in text for k_i.

than k_2, suggesting that the cleavage is easier when a near-neighboring region is previously cleaved by trypsin.

Discussion

A variety of physicochemical methods have been used to detect local and/or overall structural changes in rabbit skeletal muscle tropomyosin [see, e.g., Lehrer et al. (1981) and Talbot & Hodges (1982) for references]. In general, it seems well established that the tropomyosin molecule is not homogeneous in stability throughout the sequence but rather heterogeneous; i.e., a part of the molecule is much less stable (or more flexible) than the remainder. In particular, a segment near Cys-190 seems much more "meltable" in solution than the remainder of the molecule (Lehrer, 1978; Lehrer et al., 1981), which is very likely reflected in the flexible nature of tropomyosin in crystal forms (Phillips et al., 1979, 1980). More recent studies on the local stability of tropomyosin and other coiled-coil molecules appear to indicate that tropomyosin is made of several cooperative quasi-independent melting blocks (Potekhin & Privalov, 1982; Privalov, 1982).

The enzyme-probe method employed in this study is advantageous in localizing unstable regions otherwise very dif-

ficult to pinpoint and detect. Since 39 lysine and 14 arginine residues out of 284 (per polypeptide chain) are evenly distributed throughout tropomyosin except Lys-5–Lys-7 (Stone & Smillie, 1978), trypsin is an appropriate enzymatic probe. Derivatives of the normalized rate constants obtained in this study (dotted lines in Figure 7) indicated a thermal pretransition similar to that indicated from circular dichroism studies (Lehrer, 1978; Lehrer et al., 1981) and differential scanning calorimetric measurements (Krishnan et al., 1978; Potekhin & Privalov, 1978). This is particularly encouraging for the enzyme-probe method since the above comparison clearly suggests that (1) normalized cleavage rate constants follow structural changes *quantitatively* and (2) structural changes in solution are localized between residues 130 and 190 under the present experimental conditions. A very good correlation between enzyme susceptibility and local instability predicted from primary amino acid sequence has also been suggested (Smillie et al., 1980). Although there seem to be many potential tryptic cleavage sites in tropomyosin (~50 Lys + Arg/polypeptide chain), the number of peptides observed in this study was very small (about three major sites). One reason that some sites are more rapidly cleaved than others is that the cleavage rate in an open state depends on the presence of a particular residue within a given protein sequence. For example, Arg–Ala bonds may be cleaved much more rapidly than other combinations of the peptide sequence. A more important reason is that the segment(s) around the cleavage site must be in an "open" state or "unfolded" state at least to some extent. It should be noted that the cleavage rate constant for a coiled-coil in a *helical* state is ~0 h^{-1} units whereas that in a *random-coil* state is of the order of 10^2 h^{-1}/units or higher and that the esterase activity used for the enzyme assay has the same (or similar) temperature dependence as peptide proteolysis within a given protein sequence (Ueno & Harrington, 1984). The findings that Arg-133–Ala-134 and Arg-182–Ala-183 (and/or Arg-178–Ala-179) bonds were split much faster than Arg-21–Ala-22, Arg-101–Ala-102, and Arg-238–Ala-239 bonds [Pato & Smillie (1978) and also this study] clearly indicate that observed cleavage rates are a reflection of the conformational state in each segment. Except the Arg-182–Ala-183 bond, cleavage rates at other Arg–Ala bonds are consistent with the findings of Potekhin & Privalov (1982) from calorimetric study. They showed that a block containing the Arg-182–Ala-183 bond exhibited a melting temperature of 51–58 °C for the oxidized form of tropomyosin (fragment), whereas the present study indicates that the melting temperature for the 182–183 bond is about 32 °C for TM_S^S (Figure 7). The reasons for this discrepancy are not clear at this moment, but the present results seem to agree with fluorescence studies of tropomyosin (Graceffa & Lehrer, 1980; Lehrer et al., 1981).

A single cysteine residue (Cys-190) in rabbit skeletal muscle α-tropomyosin has attracted attention since (1) an interchain disulfide bond is easily formed (Lehrer, 1975; Stewart, 1975a; Johnson & Smillie, 1975), (2) Cys-190 is located at an appropriate position to investigate a local conformational transition in tropomyosin (Lehrer, 1978; Graceffa & Lehrer, 1980; Betteridge & Lehrer, 1983), and (3) the troponin binding site is very close to Cys-190 (Stewart, 1975b) although the binding site seems to extend over a wide region along the long axis of tropomyosin (Ohtsuki, 1975; Flicker et al., 1983). The present study extends the knowledge of the least-stable region of tropomyosin near Cys-190. It appears that the region is localized between residues 130 and 190. Previous studies showed that the disulfide destabilized the region near Cys-190 (Lehrer, 1978; Lehrer et al., 1981) due to the strain produced. The present study verifies the destabilizing effect of the disulfide bond by showing that the rate of cleavage at a point close to Cys-190, Arg-182–Ala-183 (and/or Arg-178–Arg-179), is markedly increased for oxidized tropomyosin. The results of this study show that lowering the ionic strength affects the conformational state of oxidized tropomyosin at or near Cys-190 but has very little effect on reduced tropomyosin. Graceffa & Lehrer (1980) observed a significant effect of salt on the excimer fluorescence spectrum of tropomyosin labeled with pyrene maleimide at Cys-190, which may indicate that pyrene–tropomyosin has a local conformation more similar to oxidized tropomyosin than to reduced tropomyosin. It is known that tropomyosin is markedly polymerizable in low salt at neutral pH, but this polymerizability is diminished at a very early stage of the tryptic digestion (Ooi, 1967). This rapid loss of polymerizability of tropomyosin after tryptic digestion was observed in both the reduced and oxidized forms of tropomyosin (data not shown). Therefore, aggregation of tropomyosin does not affect the results of the present study.

It is clear from the present study as well as other previous studies that the tropomyosin molecule is very flexible in solution (37 °C, μ = 0.15 M, pH 7) near its center. This flexible region lies close to the troponin-binding region, suggesting the very attractive possibility that a local conformational change (such as "melting") modulated by a Ca^{2+}-troponin system would lead to the regulation of skeletal muscle contraction in situ [see also Lehrer (1978), Lehrer et al. (1981), Potekhin & Privalov (1982), and Flicker et al. (1983)]. If such local melting occurs in the tropomyosin molecule, its end-to-end length would be expected to shorten and/or induce stress on the actin filament.

Since structural changes in coiled-coil molecules were investigated by an enzyme-probe method in this study as well as in our recent studies (Ueno & Harrington, 1984; H. Ueno and W. F. Harrington, unpublished results), it is worthwhile providing the method with a theoretical interpretation. On the basis of the reasons described below, the kinetic equation for enzymatic cleavage is considered to be analogous to that describing hydrogen exchange of proteins [see Hvidt & Nielsen (1966)].

Schematically

$$N_A \underset{k_b}{\overset{k_a}{\rightleftharpoons}} U_A \xrightarrow{k_m} P_A \qquad (1)$$

where N_A, U_A, and P_A are concentrations closed (helical) state, open (random-coil) state, and cleaved product for the region A, respectively. A solution for eq 1 to give the cleavage first-order rate constant is $k_a k_m/(k_a + k_b + k_m)$. In this case, the fraction of the open state, α, is given by $k_a/(k_a + k_b)$, assuming k_a and $k_b \gg k_m$, and the observed cleavage rate constant is simplified as αk_m. Thus, the cleavage rate of any structure is proportional to the fraction of time that the region spends in the open state when the cleavage step (k_m) is rate limiting in the enzyme-probe study. Reasons to support the present scheme are as follows: (1) A coiled-coil molecule seems to be composed of quasi-independent melting blocks (or regions) that melt in a two-state (helix $\rightleftharpoons$ coil) process (Privalov, 1982; Potekhin & Privalov, 1982). (2) A typical random-coil molecule is cleaved by an enzyme 1000 times (or more) faster than the molecule in closed state (Ueno & Harrington, 1984), suggesting that the cleavage of the molecule in the open state but not in the closed state is apparently observed. (3) A very close correlation between the enzymatic cleavage rate and the amount of the structural change detected by circular dichroism

and calorimetric studies as discussed above strongly suggests that the enzyme is following local helix $\rightleftharpoons$ coil transitions quantitatively and that the cleavage rate is proportional to the fraction of time that the region spends in open state $[k_a/(k_a + k_b)]$. (4) If the relaxation time for reequilibration of the coiled-coil molecule in helix $\rightleftharpoons$ coil transition is on the order of $10^{-4} \sim 10^{-2}$ s (Tsong et al., 1983), then the rate constants k_a and k_b are at the very least 10^5 h^{-1} for a coiled-coil molecule in solution and k_a and $k_b \gg k_m$. Therefore, the cleavage process (k_m) is rate limiting. In the present study, the structural opening in the middle region of the tropomyosin molecule, which is much more unstable thermally than the rest of the molecule, was probed by the tryptic digestion method.

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