Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys, Res. Commun. 28,* 815.
Stafford, W. (1973), Ph.D. Thesis, University of Connecticut.
Stafford, W., and Yphantis, D. A. (1972), *Biochem. Biophys. Res. Commun. 49,* 848.
Szent-Gyorgyi, A. G., Cohen, C., and Kendrick-Jones, J. (1971), *J. Mol. Biol. 56,* 239.
Twarog, B., and Mineoka, Y. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 489.

Polymerizability of Rabbit Skeletal Tropomyosin: Effects of Enzymic and Chemical Modifications†

Peter Johnson‡ and Lawrence B. Smillie*

ABSTRACT: Polymerizability of tropomyosin was unaffected by the removal of the three terminal residues 282, 283, and 284 using carboxypeptidase A. However, when residue 281 was removed, polymerizability was abolished. These results are consistent with a 9-residue molecular head-to-tail overlap in polymerized tropomyosin, in which residue 281 plays a space-filling role at the center of the overlap core. In acetylation studies, loss of polymerizability closely paralleled the extent of acetylation of lysine-7, and this residue was more susceptible to acetylation than any other. The effect of acetylation on polymerizability was probably caused not only by cleavage of salt-bridge between lysine-7 ϵ-NH_2 and residue 284 α-COOH but also by distortion of the overlap core by the *N*-acetyl group. Specific modification of methionine in tropomyosin indicated that, in addition to residue 281, methionine-8 is also involved in formation of the overlap core. Modified nonpolymerizable tropomyosins could still bind to F-actin, indicating that the head-to-tail polymerization of tropomyosin is not a prerequisite for actin binding, although the regularity of tropomyosin molecules along the actin helix is presumably disrupted.

The tropomyosin molecule is a highly helical asymmetric protein (Cohen and Szent-Gyorgyi, 1957) of molecular weight 66 000 (Woods, 1967) in which the two identical or near-identical polypeptide chains (Hodges and Smillie, 1970; Cummins and Perry, 1974) are aligned in parallel (Caspar et al., 1969) and in register (Johnson and Smillie, 1975; Lehrer, 1975; McLachlan and Stewart, 1975; Stewart, 1975). The complete amino acid sequence of one of the two major forms of rabbit skeletal tropomyosin (α-tropomyosin) has been established (Stone et al., 1975), and, on the basis of the regularities in the distributions of hydrophobic, acidic, and basic residues, it has been proposed that the molecule is a coiled-coil α-helical structure stabilized by hydrophobic and electrostatic interactions between the two polypeptide chains (Stone et al., 1975; Sodek et al., 1972; McLachlan and Stewart, 1975).

In solutions of low ionic strength, the tropomyosin coiled coils interact with each other to form head-to-tail linear aggregates (Kay and Bailey, 1960), and, from x-ray measurements (Caspar et al., 1969) and the fact that the polypeptide chains are in register, the extent of the head-to-tail molecular overlap has been calculated to be 13 Å or approximately 8–9 residues (Johnson and Smillie, 1975; Stewart, 1975). This head-to-tail arrangement of tropomyosin molecules also exists in vivo where the molecules are located along the grooves of the I filament such that each tropomyosin spans 7 actin monomers in one strand (or 14 in both strands) and specifically binds 1 unit of the troponin complex (Ebashi et al., 1969; Potter 1974; Potter and Gergely, 1974). In this assembly, the tropomyosin molecules govern the relative positioning of the troponin complexes to the actin strands (Potter and Gergely, 1974), and the arrangement of the tropomyosin molecules themselves along the I filament must be an important factor in the mechanism by which the troponin complex can regulate the actin–myosin interaction.

Because of the importance of the head-to-tail overlap between tropomyosin molecules in dictating the periodicity of the troponin complex along the I filament, we decided to investigate the head-to-tail polymerization process in detail. As the complete amino acid sequence of α-tropomyosin and the precise arrangement of the two polypeptide chains are known, we have been able to study the involvement of specific amino acid residues in the head-to-tail overlap by chemical and enzymic modifications of tropomyosin and by model building studies. These results are consistent with the molecular model proposed by McLachlan and Stewart (1975) for the 8–9 residue overlap and demonstrate the importance of both electrostatic and hydrophobic interactions between the NH_2-terminal and COOH-terminal regions of adjacent molecules.

Materials and Methods

Protein Preparations. Tropomyosin was prepared from rabbit skeletal muscle and resolved into α and β components by the method of Cummins and Perry (1973). Tropomyosin-free actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (1971).

Helix Determinations. The method of Chen et al. (1974) was used to calculate helix contents from circular dichroism spectra at 10 °C of tropomyosin preparations at concentrations

† From the Medical Research Council of Canada Group on Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. *Received September 16, 1976.* This work was supported by the Medical Research Council of Canada.

‡ Present address: Department of Chemistry, Ohio University, Athens, Ohio 45701.

of 1 mg/mL in 1 M KCl-2 mM DTT[1]-0.05 M phosphate buffer (pH 7.0).

Carboxypeptidase A Digestion of Tropomyosin. Because commercial preparations of carboxypeptidase A-DFP have significant endopeptidase activity, Worthington carboxypeptidase A-DFP was dissolved in 5 M NaCl-5 mM phosphate buffer (pH 7.5) at a concentration of approximately 2 mg/mL and incubated at 34 °C for 1 h after the addition of 1 μL of 1 M DFP in 2-propanol per mL of dilute carboxypeptidase A. After incubation with DFP, which dramatically reduced the endopeptidase activity, the enzyme was used to digest tropomyosin by a modification of the conditions of Tawada et al. (1975) in which tropomyosin at 4 mg/mL was digested in 0.1 M KCl-10 mM Tris (pH 8.0) at 34 °C, using an enzyme:substrate ratio by weight of 1:200.

Samples from the digest for amino acid analysis were pipetted into 9 volumes of 0.2 M sodium citrate buffer (pH 2.2) and then analyzed directly on a Beckman 120C amino acid analyzer in which the long column was equilibrated with lithium citrate buffers in order that the amides could be resolved (Benson, 1972).

For viscosity measurements, samples from the digest were immediately pipetted into 9 volumes of distilled deionized water so that the final solvent composition was 10 mM KCl-1 mM Tris (pH 7.5), with a protein concentration of 0.4 mg/mL. The viscosities of 1 mL aliquots were measured immediately after the dilution using a Cannon 50-A75 viscometer at 19 °C.

In actin-binding studies, formic acid was added to samples to give a 5% (v/v) formic acid concentration and the samples were then dialyzed against 5% formic acid for 48 h in order to inactivate the carboxypeptidase A (Vallee et al., 1960) and then lyophilized and redissolved in 0.1 M KCl-1 mM DTT-0.2 mM ATP-10 mM Tris-HCl (pH 8.0). Tropomyosin was combined with F-actin using 2.5 mg of actin and 0.5 mg of tropomyosin in 2.5 mL of 0.1 M KCl-1 mM DTT-0.2 mM ATP-10 mM Tris-HCl (pH 8.0) in accordance with the optimal conditions for actin-tropomyosin binding reported by Tanaka (1972). After 2 h at room temperature, the solutions were centrifuged at 100 000g for 3 h and the supernatants and precipitates lyophilized. The actin and tropomyosin components of these fractions were visualized using $NaDodSO_4$-polyacrylamide gel electrophoresis (Weber and Osborn, 1969) on a vertical slab apparatus (Perrie et al., 1973), followed by staining of the gel with Coomassie brilliant blue and scanning of the stained gel at 590 nm. The amount of each protein present was then calculated from the peak area of the gel scan by reference to standard curves constructed for each protein in the concentration range 0-50 μg/cm of gel.

Acetylation of Tropomyosin. Tropomyosin (4 mg/mL) was acetylated at room temperature in its polymerized state (in 2 mM phosphate buffer (pH 8.0)) and in its depolymerized state (in 1 M KCl-2 mM phosphate buffer (pH 8.0)) using [1-^{14}C]acetic anhydride (ICN Chemicals and Radioisotopes) of specific activity 0.6 mCi/mmol. The acetic anhydride was added as a 1% solution in acetone, and the pH of the reaction solution was maintained by the addition of 0.1 M NaOH from a pH-stat. The reaction and hydrolysis of the anhydride were complete within 15 min as determined by base uptake.

The effect of acetylation on polymerized tropomyosin was followed viscometrically, and samples of acetylated tropomyosin were diluted with 2 mM phosphate buffer (pH 8.0) to a protein concentration of 0.4 mg/mL for viscosity measurements.

Samples of carboxypeptidase A digested tropomyosin for acetylation were adjusted to pH 3.5 by the addition of 0.1 volume of 0.6 M pyridine-acetic acid buffer (pH 3.5) and these were then dialyzed against 0.06 M pyridine-acetic acid buffer (pH 3.5), containing 1 mM 1,10-phenanthroline for 24 h at 0 °C, and then against 2 mM phosphate buffer (pH 8.0), containing 1 mM 1,10-phenanthroline for a further 24 h at 0 °C before the acetylation reaction was performed.

In actin-binding studies, the acetylated protein was dialyzed against 0.1 M KCl-1 mM DTT-0.2 mM ATP-10 mM Tris (pH 8.0) and 0.5 mg of the dialyzed tropomyosin was assayed for actin-binding ability as described previously.

Identification of Modified Lysine Residues in Acetylated Tropomyosin. Acetylation of 200 mg of α-tropomyosin in 40 mL of 1 M KCl-2 mM phosphate buffer (pH 8.0) was performed at a molar ratio of acetic anhydride:lysine groups of 0:2. After the acetylation, the tropomyosin solution was dialyzed against 5 mM $CaCl_2$-0.05 M *N*-ethylmorpholine hydrochloride buffer (pH 8.0) and then digested with 2 mg of thermolysin (B grade, Calbiochem) for 18 h at 34 °C, after which the solution was lyophilized. The lyophilized material was then redissolved in 4 mL of 0.25 M pyridine-acetic acid buffer (pH 6.7) and fractionated on a Dowex 1-X2 column according to the method of Johnson and Smillie (1971). Each of the fractions obtained was then subfractionated on Technicon Chromobead-P ion-exchange resin by the method of Welinder and Smillie (1972) using gradient system I. Where necessary, further purifications of ^{14}C-labeled peptides were achieved by high-voltage paper electrophoresis at pH 6.5 or 1.8.

The amino acid sequences of the ^{14}C-labeled peptides were established by the dansyl-Edman method (Hartley, 1970), and, in peptides which contained two or more lysine residues, the locations of the acetyl groups were established by scintillation counting of the 2-anilino-5-thiazolinone derivatives of the amino-terminal residues released during the Edman degradations.

On the basis of the known electrophoretic mobilities of the acetylated peptides, pH 6.5-1.8 peptide maps were used to establish the extents of lysine group modifications under different conditions. The completed peptide maps were stained with cadmium-ninhydrin reagent (Heilmann et al., 1957), and the areas of the maps corresponding to acetylated peptides were cut out and counted in 10 mL of Scintiverse (Fisher).

Modification of Methionine Residues. Carboxamidomethylation of methionine residues in tropomyosin was performed in 0.06 M pyridine-acetic acid buffer (pH 3.5), at a protein concentration of 3 mg/mL with a threefold weight excess of iodoacetamide. After incubation at 34 °C for 72 h, the solution was dialyzed extensively against 1 mM DTT-2 mM phosphate buffer (pH 7.5), and for viscosity measurements the protein concentration was adjusted to 0.4 mg/mL. The number of modified methionines was determined by the method of Neumann et al. (1962) after lyophilization of the dialyzed carboxamidomethylated tropomyosin.

Results

Carboxypeptidase A Digestion of Tropomyosin. The release of free amino acids and the relative viscosity of α-tropomyosin during digestion by carboxypeptidase A are shown in Figure 1. Under these conditions, endopeptidase degradation of tropomyosin was negligible as evidenced by the absence of smaller

[1] Abbreviations used: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; CPK, Corey-Pauling-Koltun.

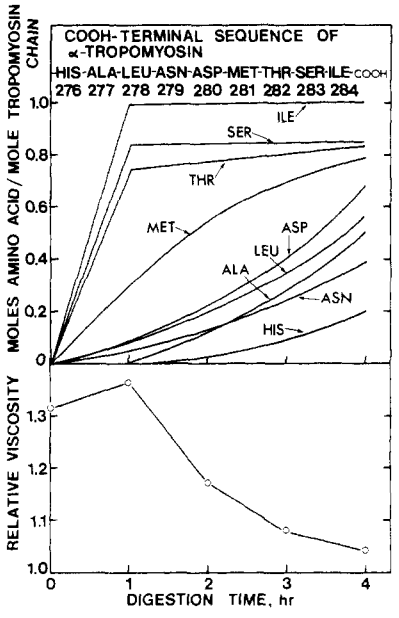

FIGURE 1: Carboxypeptidase A digestion of α-tropomyosin in 0.1 M KCl-10 mM Tris (pH 8.0) buffer. The tropomyosin concentration was 4 mg/mL and the enzyme:substrate ratio was 1:200 by weight. Relative viscosities were determined at a tropomyosin concentration of 0.4 mg/mL by dilution of the digest sample with water. The release of free amino acids was determined by direct analysis of digest samples on a Beckman 120C amino acid analyzer. The COOH-terminal amino acid sequence of α-tropomyosin is taken from Stone et al. (1975).

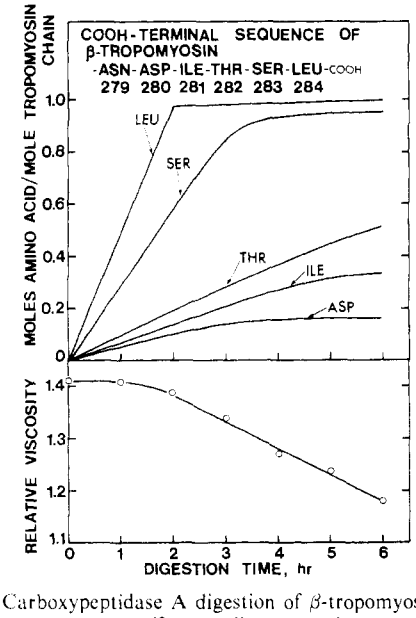

FIGURE 2: Carboxypeptidase A digestion of β-tropomyosin in 0.1 M KCl-10 mM Tris (pH 8.0) buffer. The digestion and assay conditions were identical with those in Figure 1. The COOH-terminal amino acid sequence of β-tropomyosin was determined by G. R. Stewart and L. B. Smillie (unpublished observations).

molecular weight protein bands on $NaDodSO_4$–polyacrylamide gel electrophoresis. These results show that α-tropomyosin is polymerizable even after removal of the three carboxyl-terminal residues isoleucine-284, serine-283, and threonine-282, but that the α-tropomyosin is nonpolymerizable when methionine-281 is removed.

Similar results were obtained for carboxypeptidase A digestion of β-tropomyosin (see Figure 2), except that the release of free amino acids was slower than that from α-tropomyosin,

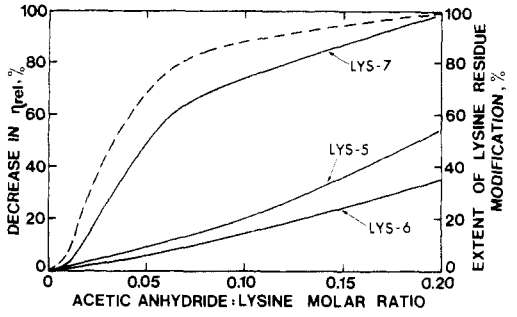

FIGURE 3: The effect of acetylation on the polymerizability and specific modification of lysine residues in α-tropomyosin. Tropomyosin (4 mg/mL) was acetylated in 2 mM phosphate (pH 8.0) buffer using [1-^{14}C]acetic anhydride at various molar ratios of acetic anhydride to total lysine ε-NH_2 groups. The relative viscosities of the acetylated tropomyosins were determined at 0.4 mg/mL by dilution of the samples with 2 mM phosphate (pH 8.0) buffer. The decrease in relative viscosity on acetylation (- - -) was expressed as a percentage of the total change taking the relative viscosity of tropomyosin acetylated at a ratio of 0.2 as 100%. Identification and quantitation (—) of the modified lysine residues were determined by isolation and analysis of peptides from thermolytic digests of the acetylated tropomyosins as described in the Materials and Methods section. For clarity, of the lysine residues which were modified more slowly than lysine-7, only the acetylation curves for lysine-5 and lysine-6 are shown. Representative data for the other modified lysines are shown in Table I.

and the initial viscosity increment after 1–2 h digestion was not seen.

The results of actin-binding studies on carboxypeptidase A digested α-tropomyosin showed that, after 3 and 4 h of digestion when the relative viscosity had been reduced to 25 and 12%, respectively, of the control, the actin-binding was still 97 and 94% of the control. These data thus show that the ability of α-tropomyosin to combine with actin is not dramatically reduced even when residues methionine-281 and aspartate-280 are removed, in contrast to the effect of removal of these residues on polymerizability.

Acetylation of α-Tropomyosin. Because the α-tropomyosin sequence has several lysine residues close to the amino-terminal end of the molecule (lysines 5, 6, 7, 12, and 15), the possible involvement of these residues in polymerization was investigated by their modification with acetic anhydride.

Polymerized α-tropomyosin was acetylated with acetic anhydride at different molar ratios of acetic anhydride to lysine ε-NH_2 groups, and, as shown in Figure 3, the α-tropomyosin was readily depolymerized even at quite low molar ratios of acetic anhydride to total lysine ε-NH_2 groups. At a molar ratio of 0.1, polymerizability was largely abolished, although actin-binding ability was unaffected. Actin-binding ability was, however, abolished when the molar ratio of acetic anhydride to ε-NH_2 lysine groups was increased to 0.2.

Identification of the lysine ε-NH_2 groups in depolymerized tropomyosin susceptible to acetylation was determined at a molar ratio of 0.2 acetic anhydride:lysine ε-NH_2 group. Under these conditions, both polymerizability and actin-combining ability were abolished, although the gross secondary structure of the molecule was unaffected in terms of its helix content calculated from circular dichroism measurements. Tables I and II show the compositions and sequences of the acetylated peptides from a thermolytic digest of this acetylated α-tropomyosin, and Table III shows the susceptible lysine residues as they are numbered in the α-tropomyosin sequence.

The extent of the acetylation of each lysine ε-NH_2 group in polymerized tropomyosin was determined as a function of the molar ratio of acetic anhydride:lysine ε-NH_2 groups by the

TABLE I: Acetylated Peptides Isolated from a Thermolytic Digest of Acetylated α-Tropomyosin.

Peptide[a]	pH 6.5 Mobility	Net Charge at at pH 6.5	Yield[b] (nmol)	Composition and sequence[c]
I-1	+0.38	+1	290	Ile-Ac·Lys-Lys-Ac·Lys 0.9 1.0 1.0 1.0
I-2A	+0.41	+1	290	Ala-Ac·Lys-His 1.0 1.0 1.0
I-2B	+0.38	+1	110	Ile-Lys-Ac·Lys-Ac·Lys 0.8 1.0 1.0 1.0
I-3	+0.41	+1	450	Leu-Gln-Ac·Lys-Lys 1.0 1.0 1.0 1.0
I-4	+0.41	+1	270	Val-(Ala,Arg,Ac·Lys) 0.9 0.9 1.0 1.0
I-5	0	0	280	Val-(Thr,Ac·Lys) 0.7 0.7 1.0
I-6	+0.74	+2	706	Ile-Lys-Lys-Ac·Lys 0.8 1.0 1.0 1.0
II-1	0	0	300	Leu-Ac·Lys 0.9 1.0
II-2	0	0	460	Ile-(Ac·Lys,Ac·Lys,Ac·Lys) 0.8 1.0 1.0 1.0
III-1	−0.71	−2	650	Leu-(Asp,Ac·Lys,Glu,Asn,Ala) 0.9 1.2 1.0 1.0 1.2 1.0
III-2	−0.45	−1	75	Leu-(Glu,Ac·Lys,Ser) 0.9 1.2 1.0 1.0
III-3A	−0.49	−1	250	Leu-(Ser,Asp,Ac·Lys) 1.2 0.9 1.1 1.0
III-3B	−0.55	−1	75	Ala-Glu-Ac·Lys 0.9 1.1 1.0
III-3C	−0.51	−1	90	Leu-(Ac·Lys,Asp) 1.0 1.0 1.1
III-4	−0.49	−1	1100	Leu-(Ac·Lys,Glu) 1.1 0.9 1.0
III-5	−0.44	−1	550	Ala-(Glu,Ac·Lys,Tyr) 0.9 1.1 1.0 0.6
III-6	0	0	190	Met-Ac·Lys 1.2 1.0
III-7	−0.67	−1	180	Gln-(Glu,Ac·Lys) 1.0 1.0 0.7

[a] Peptide nomenclature: the Roman numeral indicates the Dowex 1-X2 fraction in which the peptide was recovered, the Arabic numeral indicates the ^{14}C-containing peptide fraction from Chromobead-P, and the letter denotes a further purification at pH 6.5 or 1.8. [b] From a digest of 6 μmol of ^{14}C-acetylated α-tropomyosin. [c] Ac·Lys denotes ε-*N*-acetyllysine. See Table II for assignments of Ac·Lys residues in peptides with two or more lysine residues.

TABLE II: Assignments of Acetyl Groups in Peptides Containing More Than One Lysine Residue.[a]

Peptide	% Distribution of total ^{14}C counts Residue number 1	2	3	4	Acetyl group assignment
I-1	7	52	4	37	Ile-Ac·Lys-Lys-Ac·Lys
I-2A	8	12	40	40	Ile-Lys-Ac·Lys-Ac·Lys
I-3	9	7	60	24	Leu-Gln-Ac·Lys-Lys
I-6	9	7	20	64	Ile-Lys-Lys-Ac·Lys

[a] For experimental details, see Materials and Methods. The evidence for the amino acid sequences of these peptides is presented in Table I.

TABLE III: Lysine Residues of α-Tropomyosin Modified by Acetic Anhydride at a 0.2 Molar Ratio of Acetic Anhydride:Lysine ε-NH_2 Groups.[a]

Lysine residue no. in sequence	Peptide(s) containing labeled lysine	% recovery of labeled lysine (from 6 μmol of acetylated α-tropomyosin)
5	I-1, II-2	13
6	I-2B, II-2	10
7	I-6, I-2B, I-1, II-2	26
12 or 266	II-1	6
15	III-1	11
48	I-3	8
65	III-3C	2
70	III-7	4
118 or 213	III-3B	1
128	III-6	3
149 or 233	III-4	18
152	I-2A	5
168	I-4	5
213	III-5	9
231	III-3A	4
248	I-5	5
251	III-2	1

[a] For experimental details of the isolation of the peptides obtained from a thermolytic digest of the acetylated tropomyosin, see Materials and Methods. The labeled lysine residues were assigned on the basis of sequence information of the thermolytic peptides and the known amino acid sequence of α-tropomyosin (Stone et al., 1975).

peptide mapping technique described in the Materials and Methods section. Figure 3 shows the extents of acetylation of lysines 5, 6, and 7 (for clarity, other slowly modified lysines have been omitted and their extents of acetylation are seen in Table III). These results show that the acetylation of lysine-7 closely paralleled the depolymerization caused by acetylation, and that this lysine was acetylated in preference to other lysine residues. It was therefore concluded that depolymerization of α-tropomyosin caused by acetylation was a result of the modification of lysine-7 ε-NH_2 group. These results also showed that the patterns of lysine acetylation in both polymerized and depolymerized tropomyosin were similar.

A similar acetylation experiment was done using α-tropomyosin which had previously been digested with carboxypeptidase A to remove isoleucine-284, serine-283, and threonine-282, but which still retained its ability to polymerize. Figure 4 shows that, in this case, the depolymerization of the carboxypeptidase A digested, acetylated α-tropomyosin was not complete, although lysine-7 ε-NH_2 group was still rapidly and completely acetylated. As molecular model building studies show that, in a head-to-tail overlap, lysine-7 ε-NH_2 can salt-bridge to isoleucine-284 α-COOH in unmodified α-tropomyosin or to methionine-281 α-COOH in carboxypeptidase A digested α-tropomyosin, these results indicate that the effect of acetylation on polymerizability is not directly due to rupture of this salt-bridge.

Methionine Modification of Tropomyosin. The effect of specific chemical modification of methionyl side-chains on the polymerizability of tropomyosin was investigated because of the proximity of several methionine residues (methionines 1, 8, and 10, and in α-tropomyosin, residue 281) to the putative head-to-tail overlap. That specific modification of methionine

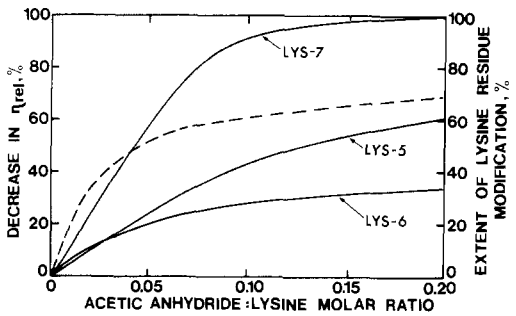

FIGURE 4: The effect of acetylation on the polymerizability and specific lysine residue modification of polymerizable carboxypeptidase A digested α-tropomyosin. Tropomyosin (4 mg/mL) was digested with carboxypeptidase-A at an enzyme:substrate ratio of 1:200 by weight for 1 h at 34 °C in 0.1 M KCl-10 mM Tris (pH 8.0) buffer. Under these conditions, the tropomyosin retained its polymerizability after removal of the three COOH-terminal residues (282-284). The digested polymerizable tropomyosin was then acetylated at various ratios of acetic anhydride:total lysine ϵ-NH_2 groups, and the polymerizability (- - -) and extent of lysine residue modification (—) were determined as in Figure 3. Except for lysine-5 and lysine-6, the acetylation curves of the slower reacting lysines have been omitted for clarity.

was achieved under the chosen conditions was evidenced by inspection of the amino acid analyses of the hydrolysates of performic acid oxidized carboxamidomethylated tropomyosins, particularly with respect to the histidine contents and the absence of carboxymethylcysteine sulfone in the hydrolysates. Table IV shows that, with both α- and β-tropomyosins, inhibition of polymerization occurs on modification of methionine residues by carboxamidomethylation, although the helical contents of these tropomyosins were not affected as determined from circular dichroism experiments.

As β-tropomyosin has an isoleucine residue at position 281 (G. R. Stewart and L. B. Smillie, unpublished observations) rather than a methionine as in α-tropomyosin, these results suggest that the head-to-tail overlap is perturbed by the modification of a methionine other than methionine-281. Model-building studies suggest that the most likely methionine to play a crucial role in the head-to-tail overlap (other than methionine-281) is methionine-8.

Discussion

In previously reported model-building studies of the putative head-to-tail overlap in the tropomyosin polymer, McLachlan and Stewart (1975) proposed that a nine-residue overlap between the broad faces of the NH_2- and COOH-terminal coiled coils gives a favorable series of hydrophobic interactions which could stabilize the head-to-tail polymer.[2] In addition to these interactions, electrostatic salt-bridge interactions between the two coiled coils can occur, and the importance of such salt-bridges is evident from the fact that the polymerization of tropomyosin is abolished at high ionic strength (Tsao et al., 1951). Our model-building studies using CPK space-filling components and the results of our chemical modifications support the idea of a nine-residue head-to-tail overlap and emphasize the importance of certain amino acid residues in the overlap. Although we confirm most of the interactions mentioned by McLachlan and Stewart, we find that optimal packing occurs when the lysine-5 ϵ-NH_2 on the broad face on an overlap side is salt-bridged to aspartate-280 β-COOH also on the broad face of the other overlap side so that an internal

[2] For a schematic diagram illustrating the hydrophobic and ionic interactions in the postulated head-to-tail overlap region, the reader is referred to Figure 4 of the paper by McLachlan and Stewart (1975).

TABLE IV: Effect of Carboxamidomethylation of Methionine Residues on the Polymerizability of α- and β-Tropomyosins.[a]

	No. of methionine residues carboxamidomethylated	% of control rel viscosity
α-Tropomyosin[b]	5.7	10
β-Tropomyosin[c]	4.5	4

[a] For experimental details, see Materials and Methods. [b] The α-tropomyosin sequence has 6 methionines (Stone et al., 1975). [c] The β-tropomyosin sequence has 8 methionines (G. R. Stewart and L. B. Smillie, unpublished observations).

salt-bridge is formed. In this arrangement, lysine-6 ϵ-NH_2 (which McLachlan and Stewart suggested is salt-bridged to aspartate-280 β-COOH) can then be externally salt-bridged to aspartate-275 β-COOH.

In agreement with the report of Tawada et al. (1975), we have found that extensive carboxypeptidase A digestion of tropomyosin results in loss of polymerizability, but we have also been able to find conditions under which removal of the three COOH-terminal residues (284, 283, 282) can occur without loss of polymerizability. Our model-building studies suggest that the reason why polymerization is possible even after removal of residues 282-284 is that the essential hydrophobic interactions stabilizing the head-to-tail overlap are maintained and that a salt-bridge between lysine-7 ϵ-NH_2 and the new α-COOH group (from methionine-281) can be formed. On removal of methionine-281 by carboxypeptidase A, the hydrophobic core of the overlap region is disrupted because of the hole generated by the removal of the relatively bulky methionyl side chain which appears to play an important space-filling role in the center of the head-to-tail overlap region. In β-tropomyosin, residue 281 is isoleucine rather than methionine, and both model-building and carboxypeptidase A digestion suggest that, in β-tropomyosin, this isoleucine plays a similar space-filling role to methionine-281 in α-tropomyosin and that its removal causes disruption of the hydrophobic interior of the overlap region.

A consistent feature of the carboxypeptidase A digestion of α-tropomyosin is the significant increase in viscosity associated with the removal of residues 282-284. Although the reasons for this are not clear, it is possible that removal of these residues may decrease the packing strain of the overlap region without affecting any of the important interactions between the coiled coils. Such a decrease in packing strain would presumably result in an enhanced polymerization and an increased viscosity.

Acetylation studies clearly indicated that the specific modification of lysine-7 ϵ-NH_2 abolishes the head-to-tail overlap and that this side chain is more susceptible to acetylation than any other lysine in tropomyosin, possibly because it has a lower pK than any of the other lysine side chains. Although lysine-7 ϵ-NH_2 has been implicated in salt-bridging to the α-COOH of the overlapping tropomyosin in the head-to-tail arrangement, it seems unlikely that the rupture of a single salt-bridge is solely responsible for the depolymerization. Inspection of the CPK model suggests that the acetyl group introduced onto lysine-7 could sterically interfere with the formation of the hydrophobic overlap core, and experimental evidence that the acetyl group is involved in the disruption of interactions other than the salt-bridge is suggested from the acetylation study of carboxypeptidase A digested α-tro-

pomyosin. This work showed that acetylation of lysine-7 ϵ-NH_2 did not cause complete depolymerization of the carboxypeptidase A digested tropomyosin, and one interpretation of this result is that the acetyl group is able to locate on the broad face of the NH_2-terminal coiled coil in the region vacated by residues 282–284 without distorting the hydrophobic overlap core. In the case of the acetylation of undigested α-tropomyosin, the fitting of residues 282–284 to the NH_2-terminal broad face may be perturbed by the presence of the acetyl group, and this distortion may then be transmitted to the hydrophobic core, resulting in destabilization and depolymerization.

Modification of methionine residues in α- and β-tropomyosins showed that there are methionine residues in both types of tropomyosin which are located near the NH_2- or COOH-terminal overlap regions and whose modification inhibits polymerizability. The CPK models of the overlaps of these tropomyosins show that both of the methionine-1, one of the methionine-8, both of the methionine-10, and in α-tropomyosin one of the methionine-281 residues are externally placed around the coiled coils. Thus, in α-tropomyosin, there are two internal methionine residues in the overlap core (a methionine-8 and a methionine-281), whereas in β-tropomyosin, there is a single internal methionine (methionine-8), as residue 281 is isoleucine. Although identification of the modified methionines has not been attempted, it seems likely that inhibition of polymerizability occurs in both α- and β-tropomyosins as a result of the modification of the internally placed methionine residues. This would result in the formation of positively charged bulky *S*-carbamoylmethylmethionine groups which would prevent the tight packing necessary for overlap core formation. As this inhibition of polymerization occurs with β-tropomyosin, this would mean that methionine-8 is the modified residue, whereas, in α-tropomyosin, modification of either or both of the internal residues methionine-8 and methionine-281 would result in inhibition of polymerization.

The modification studies on tropomyosin have shown that, under conditions in which polymerizability of tropomyosin is inhibited, the actin-binding ability is retained, although our assay does not measure the strength (i.e., association constant) of binding. The separateness of actin-binding ability and polymerizability in the tropomyosin molecule must mean that, when nonpolymerizable tropomyosin is bound to actin, the strict periodicity of the tropomyosin molecules along the actin double helix is absent because of the loss of the ordering effect of the tropomyosin head-to-tail polymerization. As a result of this disorganization, the precise stoichiometry and geometry of the 7 actins:1 tropomyosin:1 troponin complex of the I filament would be less effective as a contractile unit because the irregularly spaced misaligned tropomyosin–troponin complexes would in many cases be unable to regulate the actin–myosin interactions. These conclusions therefore emphasize the importance of the tropomyosin head-to-tail overlap as a crucial feature of the I-filament structure.

Following submission of this manuscript, a paper by Ueno et al. appeared describing the effects of carboxypeptidase A digestion on the polymerizability of tropomyosin. Their conclusion that polymerizability is lost by the removal of residues 282, 283, and 284 is contrary to the results reported herein. However, their results are difficult to interpret since unfractionated tropomyosin (a mixture of α and β) was employed and the time course of release of the amino acids was not presented. Our results show that the rate of release of residues 282 and 281 from β-tropomyosin by carboxypeptidase A is much slower than from α-tropomyosin as is the rate of decrease of the relative viscosity.

Acknowledgments

We thank Mr. M. Nattriss for the amino acid determinations and Mr. K. Oikawa for the circular dichroism measurements.

References

Benson, J. V. (1972), *Anal. Biochem. 50,* 477.
Caspar, D. L. D., Cohen, C., and Longley, W. (1969), *J. Mol. Biol. 41,* 87.
Chen, Y.-H., Yang, J. T., and Chan, K. H. (1974), *Biochemistry 13,* 3350.
Cohen, C., and Szent-Gyorgyi, A. G. (1957), *J. Am. Chem. Soc. 79,* 248.
Cummins, P., and Perry, S. V. (1973), *Biochem. J. 133,* 765.
Cummins, P., and Perry, S. V. (1974), *Biochem. J. 141,* 43.
Ebashi, S., Endo, M., and Ohtsuki, I. (1969), *Q. Rev. Biophys. 2,* 351.
Hartley, B. S. (1970), *Biochem. J. 119,* 805.
Heilmann, J., Barollier, J., and Watzke, E. (1957), *Hoppe-Seyler's Z. Physiol. Chem. 309,* 219.
Hodges, R. S., and Smillie, L. B. (1970), *Biochem. Biophys. Res. Commun. 41,* 987.
Johnson, P., and Smillie, L. B. (1971), *Can. J. Biochem. 49,* 1083.
Johnson, P., and Smillie, L. B. (1975), *Biochem. Biophys. Res. Commun. 64,* 1316.
Kay, C. M., and Bailey, K. (1960), *Biochim. Biophys. Acta 40,* 149.
Lehrer, S. S. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3377.
McLachlan, A. D., and Stewart, M. (1975), *J. Mol. Biol. 98,* 293.
Neumann, N. P., Moore, S., and Stein, W. H. (1962), *Biochemistry 1,* 68.
Perrie, W. T., Smillie, L. B., and Perry, S. V. (1973), *Biochem. J. 135,* 151.
Potter, J. D. (1974), *Arch. Biochem. Biophys. 162,* 436.
Potter, J. D., and Gergely, J. (1974), *Biochemistry 13,* 2697.
Sodek, J., Hodges, R. S., Smillie, L. B., and Jurasek, L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3800.
Spudich, J. A., and Watt, S. (1971), *J. Biol. Chem. 246,* 4866.
Stewart, M. (1975), *FEBS Lett. 53,* 5.
Stone, D., Sodek, J., Johnson, P., and Smillie, L. B. (1975), *Fed. Eur. Biochem. Soc. Meet., [Proc.] 31,* 125.
Tanaka, H. (1972), *Biochim. Biophys. Acta 278,* 556.
Tawada, Y., Ohara, H., Ooi, T., and Tawada, K. (1975), *J. Biochem. (Tokyo) 78,* 65.
Tsao, T. C., Bailey, K., and Adair, G. S. (1951), *Biochem. J. 49,* 27.
Ueno et al. (1976), *J. Biochem. (Tokyo) 80,* 283.
Vallee, B. L., Rupley, J. A., Combs, T. L., and Neurath, H. (1960), *J. Biol. Chem. 235,* 64.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Welinder, K. G., and Smillie, L. B. (1972), *Can. J. Biochem. 50,* 63.
Woods, E. F. (1967), *J. Biol. Chem. 242,* 2859.