Modification of Lys-237 on Actin by 2,4-Pentanedione

ALTERATION OF THE INTERACTION OF ACTIN WITH TROPOMYOSIN*

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It has been possible to specifically label rabbit skeletal muscle actin at Lys-237 with 2,4-pentanedione, producing an enamine. This reaction can be reversed with hydroxylamine. This reaction can be reversed with hydroxylamine. This reaction can be reversed with hydroxylamine. This reaction can be reversed with hydroxylamine. This reaction can be reversed with hydroxylamine. This reaction can be reversed with hydroxylamine. This reaction can be reversed with hydroxylamine. The modification is still controversial. The most widely accepted theory that offers explanation for Ca2+ regulation through Tn is the steric blocking model proposed by Huxley and co-workers (3-5), based upon x-ray diffraction and three-dimensional reconstitution of electron micrographs of myosin head-decorated thin filaments (6, 7). According to this model, Tn-linked regulation of muscle contraction involves reversible Ca2+ binding to TnC (8) which causes conformational changes in the Tn molecule (9). In the absence of Ca2+, TnI is bound to actin, and Tm moves deeper into the grooves between the actin strands, exposing the myosin-binding sites on actin (3-5), thus preventing the interaction between actin and myosin. Ca2+ binding to Tn causes conformational changes in TnC which are transmitted to TnI, relieving its interaction with actin, and Tm moves deeper into the grooves between the actin strands, exposing the myosin-binding sites, thus permitting activation of contraction and ATP hydrolysis.

On the other hand, combining results from (a) binding and kinetic measurements on S-1, S-1-ADP and S-1-ATP interactions with regulated and unregulated actin (at low ionic strength) and (b) structural studies on the conformational states of the myosin heads relative to actin, Eisenberg and Greene (10), Chalovich and Eisenberg (11), and Hill et al. (12) presented an alternative allosteric mechanism for actomyosin ATPase control which contradicts a simplified steric blocking model. As recently illustrated (13), this model suggests that, in the presence of Ca2+, regulated actin exists in a strong form that binds S-1-ATP weakly at a "90°" angle, whereas S-1-ADP binds strongly at a "45°" angle. The transition of S-1 from the weak binding 90° state to the strong binding 45° state is assumed to occur rapidly and is associated with a rapid release of Pi. Rapid occurrence of this transition allows muscle contraction to occur. In the absence of Ca2+, Tm is suggested to assume a different position on the actin filament.

Tn1 together with Tm in the thin filament form a regulated actin thin filament at a molar ratio of actin:Tm:Tn of 7:1:1, respectively (1). Tn consists of three different subunits (1): TnC, the Ca2+ binding subunit; TnT, the Tm-binding subunit, and TnI, the inhibitory subunit of actomyosin ATPase. It is through Tn that Ca2+ released from the sarcoplasm reticulum into the sarcoplasm regulates contraction by controlling actin-myosin interaction (for review, see Ref. 2). The precise mechanism, however, by which Tn regulates muscle contraction is still controversial. The most widely accepted theory that offers explanation for Ca2+ regulation through Tn is the steric blocking model proposed by Huxley and co-workers (3-5), based upon x-ray diffraction and three-dimensional reconstitution of electron micrographs of myosin head-decorated thin filaments (6, 7). According to this model, Tn-linked regulation of muscle contraction involves reversible Ca2+ binding to TnC (8) which causes conformational changes in the Tn molecule (9). In the absence of Ca2+, TnI is bound to actin, and Tm moves deeper into the grooves between the actin strands, exposing the myosin-binding sites, thus permitting activation of contraction and ATP hydrolysis.

The contraction of vertebrate skeletal muscle involves interaction between the thin filaments, consisting mainly of actin, and the thick filaments, consisting primarily of myosin.

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†* The abbreviations used are: Tn, tropomysin; Tm, tropomyosin; HMM, heavy meromyosin; EGTA, ethylene glycol bis(β-aminooxyethyl) ether-N,N,N′,N′-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; pCa, negative logarithm of free calcium concentration; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; S-1, myosin fragment 1; actin-HMM Mg2+-ATPase, the actin activation of the Mg2+-stimulated heavy meromyosin ATPase activity; μ, ionic strength.
A Functionally Critical Residue (Lys-237) in Actin

filament, forming the weak state of the regulated actin which binds weakly to both S-1-ADP and S-1-ATP. In this form, the release of P_i and the associated change of S-1 from the 90° state to the 45° state is inhibited by Tm and thus occurs very slowly, causing muscle to relax.

Because of the importance of actin in muscle and nonmuscle cell (14) contraction, considerable effort has been made to elucidate the detailed structure of the molecule (15, 16) and to identify regions of the molecule that are involved or affected by its diverse interactions. A variety of different approaches have already been used to investigate actin amino acid residue function and topological location. Chemical modification studies have directly implicated His-40 (17) and Tyr-53 (18) in actin polymerization and Arg-95 in Tm-binding (19), and a variety of other studies have examined the availability of various actin amino acid residues, notably cysteine (20-23) and lysine (23-26), to chemical modification in the presence and absence of interacting proteins.

In the present studies, as an extension of the chemical modification approach, we have investigated the reaction of actin with the reagent 2,4-pentanedione (27) under conditions in which only a single lysine residue is modified, and we have discovered that this modification of Lys-237 affects the way in which Tm interacts with actin. Studies on the interactions of the modified actin with Tm and with Tn and Tn-cal (relaxed (absence of Ca2+) and activated (presence of Ca2+) states of muscle), and on the ability of the actin in these complexes to interact with HMM, revealed that, under physiological ionic conditions, it is possible to produce three Tm-actin states: off (F-actin-Tm), on (modified F-actin-Tm), and "potentiated" (modified F-actin-Tm-Tn).

**EXPERIMENTAL PROCEDURES**

### Materials

2,4-Pentanedione (Gold Label) was purchased from Aldrich and used without further purification. \([14C]\)Acetic anhydride with a specific activity of 8.2 Ci/mol was a product of New England Nuclear. All other reagents used were of analytic grade.

### Preparations of Muscle Proteins

Rabbit skeletal muscle actin and Tm were prepared as described by Zot and Potter (28) and by Smillie (29), respectively. Myosin was prepared from rabbit back muscle and HMM as described by Weeds and Pope (31). Tn from rabbit skeletal muscle was prepared by the method of Potter (32). F-actin in 10 mM MOPS, pH 7.0, 40 mM KCl was stored in liquid nitrogen and used within 3 weeks after thawing, during which it was stored at 0°C in appropriate buffers. For each experiment, Tm was freshly reconstituted (in 100 mM KCl, 0.2 mM DTT, and 10 mM MOPS, pH 7.0) from lyophilized Tm powder (stored at -20°C), and dialyzed against 2-liter changes in the C buffer (10 mM MOPS, pH 7.0, 100 mM KCl, 0.2 mM DTT, 0.2 mM ATP, 0.005% NaN3). Aggregates were then removed by centrifugation in a Beckman 50 Ti rotor at 100,000 g for 1 h at 4°C. The protein was stored at 0°C and used within a 2-3-day period. All protein stock solutions contained 1.5 mM NaN3.

### Preparation of \([14C] \text{C}2,4\text{-Pentandione}

The preparation of \([14C] \text{C}2,4\text{-pentandione was performed as described previously (33), except that \([14C] \text{C}aetic anhydride was the source of the radiolabel. The purity of the product, which had a specific activity of 43.8 \times 10^3 \text{cpm/mol}, was verified by Fourier transform NMR spectroscopy on a JEOL JNM-FX90Q spectrometer.}

### Modification of Actin and Actin-Tm Complex with 2,4-Pentandione

Monomeric G-actin at 1.0 mg/ml was treated with a 200-fold molar excess of 2,4-pentandione (labeled or unlabeled) to actin lysine content for 10 h (unless otherwise stated) at 16°C (using a shaking circulating H2O bath) in G buffer which contained 0.2 mM DTT, 0.2 mM ATP, 0.005% NaN3, 5 mM MOPS, pH 7.0. These conditions were necessary to minimize the possible modification of arginine residues (27). During the modification, the reagent 2,4-pentandione was added slowly to actin with gentle stirring and the pH was carefully adjusted to pH 7.0 with 0.5 M KOH. Removal of excess reagent was performed by exhaustive dialysis against the G buffer. The 2,4-pentandione-treated G-actin was then centrifuged for 1.5 h at 100,000 \times g at 4°C to remove any protein aggregates.

Similar modification experiments were performed on F-actin (1.0 mg/ml) in G buffer (5 mM NaHCO3, pH 8.0, 100 mM KCl, 0.2 mM DTT, 3.2 mM ATP, 0.005% NaN3) except that the reaction time was reduced to 5 h.

In addition, a complex formed between F-actin and Tm was also treated with 2,4-pentandione. For this purpose, 10 mg of F-actin (at 1 mg/ml) and 5 mg of unlabeled or 3.7 mg/ml of labeled Tm buffer were reconstituted at an actin:Tm molar ratio of 7:1. The protein mixture was allowed to incubate for 3 h at 22°C after which it was treated with 2,4-pentandione at 200-fold molar excess of the reagent to all lysine amino groups in the complex for 10 h. Several modification experiments were also performed at pH 8.0 with NaHCO3 (at 5 mM) substituting for the 10 mM MOPS in the C buffer.

Following removal of excess reagent by dialysis against several changes of the C buffer (pH 7.0 or 8.0), F-actin was dissociated from the complex by centrifugation at 100,000 \times g after addition of KCl to the supernatant to a concentration of 0.8 M (24), as a control, F-actin (35 mg) sample was also treated with 0.6 M KCl to check for the stability of the modified product. The actin was then suspended and homogenized in the G buffer and dialyzed against several changes of the same buffer. Any remaining aggregates were removed by centrifugation at 100,000 \times g for 1 h. Actin samples from the supernatant were then analyzed spectrophotometrically (measuring absorbance at 280 nm) for any formation of enamine products and acid hydrolyzed for amino acid analysis to determine whether the arginine residues were modified by 2,4-pentandione (27).

**Determination of the Extent and Nature of Reacting Residues in the 2,4-Pentandione-treated Actin**

**By Amino Acid Analysis** — The extent of any possible modification of arginine residues and any destruction of other residues was determined by amino acid analysis on 2,4-pentandione-treated and untreated actin samples. For this purpose, lyophilized actin samples (1.0 mg each) were hydrolyzed in 0.3 ml of constant boiling 6 N HCl in evacuated seal tubes for 24 h at 110°C. After removal of the HCl under reduced pressure, sample residues were dissolved in 100 \( \mu \)l of citrate buffer, pH 7.2, clarified for 2 min in a Beckman Microfuge, and used on a Beckman 119CL amino acid analyzer equipped with a Spectroscopy System I computing integrator.

**By Light Absorption** — Ultraviolet spectra of 2,4-pentandione-treated actin and untreated actin were scanned in the range of 350-270 nm on a Beckman Model 35 spectrophotometer set at 30°C against a reference 1.0-ml cuvette containing the same actin buffer. The enamine formed by modification of the amino groups of lysine residues in the actin molecule by 2,4-pentandione was quantified using an extinction coefficient of 2.1 \( \times 10^4 \) cm\(^{-1}\) \( \mu \)l\(^{-1}\) at a \( \lambda_{max} \) of 312 nm. The stability of the enamine product in actin stored at 0°C and at pH 7.0 was determined by periodic scanning of the modified protein.

**By Radiolabeling Using Radioactive 2,4-Pentandione** — 55 mg of G-actin labeled with \([14C] \text{C}2,4\text{-pentandione was diazylated against 0.2 mM ATP, 0.2 mM DTT, 5 mM NaHCO3, pH 8.0, and after denaturation by incubation at 100°C for 3 min, the protein was digested for 18 h at 37°C with a mixture of trypsin and Staphylococcus aureus V8 protease each at an actin-enzyme ratio of 50 by weight. During the digestion, a second addition of the enzymes was also made after 2 h of digestion. The digest was lyophilized and the material was electrophoresed in 0.1 M ammonium carbonate, pH 9.0, on Whatman No. 1 paper for 45 min at 3000 V in a Varsol-cooled Gilson Model D electrophorator. Side strips were removed from the electropherogram to locate radioactive regions by scintillation spectrophotometry (in Aquasol, New England Nuclear) on a Beckman LS-800 liquid scintillation spectrometer using the proper \( \delta \) channel. The single radioactive band thus located was then subjected to descending paper chromatography in methanol/polyethylene/water (20:1:5 by volume, adjusted to pH 9.0 with ammonium hydroxide). The single radioactive band located on the scintillation coproduct strip was then re-electrophoresed at pH 9.0 before elution with ammonium carbonate pH 9.0 buffer and lyophilization. Amino acid analysis and N-terminal identification of the peptide carrying the radioactive label was performed as described previously (19, 35) and the mobility of
the peptide at pH 6.5 was determined by the method of Offord (36) after incubation of the peptide in pyridine acetic acid buffer, pH 3.5, for 4 h at 45 °C in order to degrade the enamine group in the peptide (27). Combining data from amino acid analysis, N-terminal analysis and electrophoretic mobility of the deblocked peptide and the known proteolytic specificities of the digestion enzymes, the isolated peptide was then identified in the previously published actin primary structure (15).

Reversal of Modification by Hydroxylamine Treatment of 2,4-Pentanedione-treated Actin

Samples of 3 ml each of modified and control G-actin samples (at 1.0 mg/ml in G buffer) were treated with hydroxylamine (0.1 M NH₂OH.HCl in H₂O) to a final reagent concentration of 6.25 × 10⁻³ M at pH 6.5. The hydroxylamine was added dropwise to each of the actin samples with constant gentle stirring and the pH was maintained at 6.5 by addition of 0.1 N KOH. The reaction was allowed to go for 1 h at 22 °C with constant shaking. Excessive hydroxylamine was removed by dialysis against the appropriate buffer.

For treatment of modified F-actin and control F-actin with hydroxylamine, the proteins were first dialyzed against 10 mM MOPS, pH 7.0, 100 mM KCl, 0.2 mM DTT, 0.2 mM ATP, 0.005% NaN₃. Otherwise, the procedure was identical to that described above for modified and control G-actin.

Circular Dichroism Spectroscopy

Measurements of the ultraviolet circular dichroism spectra of unmodified and 2,4-pentanedione-treated actin were performed with a Cary Model 61 spectropolarimeter calibrated with 10-camphorsulfonic acid. Spectra of each actin sample (0.48 mg/ml in G buffer) were recorded after the samples had remained in the 1-mm path length cylindrical cell for 1 h to minimize spurious time-dependent dichroism effects (37). The results were expressed as mean residue ellipticity values at given wavelengths [θ]nin/nin based on a mean residue weight of 115, in units of degrees cm² dmol⁻¹. Any excess ellipticity contributed by the unbound nucleotide in the G buffer was subtracted to give the curves for the actin-nucleotide complexes. CD spectra in the region below 210 nm had relatively high noise to signal ratio and, therefore, were not recorded. The temperature of the sample was controlled at 25 °C by means of a cell holder attached to a circulating constant temperature bath.

Viscosity Measurements

Reduced viscosities of actins (at 1.0 mg/ml in C buffer) were measured using a Cannon-Manning Semi-Micro calibrated viscosimeter (size 100, No. B917) of 1.0-ml charge capacity. Reduced viscosity was calculated using the relationship: 

\[ \eta = \frac{1}{C} \left( \frac{t_s}{t_b} - 1 \right) \]

where \( t_s \) is the flow time (in seconds) of each actin sample, \( t_b \) is the flow time of the sample buffer (60 μl), \( C \) is the concentration of actin in milligrams/ml. All flow measurements were performed at 25 °C using a waterbath.

Protein-Protein Interaction Assays

2,4-Pentanedione-treated and untreated G-actin samples were first polymerized by overnight dialysis against the pH 7.0 C buffer, described above, at 4 °C. Also, 2,4-pentanedione-treated and untreated F-actin samples (originally in the pH 8.0 F buffer) were dialyzed against the pH 7.0 C buffer. Similarly, the interactions of each of the above actins with Tm alone and with Tm and Tn were analyzed by centrifugation assays. Actin was mixed with (a) Tm and a 2-ml volume of the pH 7.0 C buffer. The samples were then centrifuged at 100,000 × g for 20 min at 23 °C on a Beckman analytical centrifuge. Controls of Tm and Tn components without actin were performed to check that the components did not sediment in the solvent system used. In the absence of Ca²⁺, the C buffer contained 1 mM EGTA. When Ca²⁺ was present, the pCa in the same buffer was maintained at 4.0 using the computer program described by Robertson et al. (38) for calculation of free Ca²⁺ concentrations. In the assays including Ca²⁺ and Mg²⁺ ions, the free Mg²⁺ concentration was 5.0 mM and the KC1 concentration was reduced to 85 mM to maintain the same ionic strength in the C buffer.

The actin-containing pellets were then suspended each in 2 ml of 1.0% SDS, 0.5% β-mercaptoethanol for overnight incubation at 22 °C and homogenized on the following day, and the supernatants were dialyzed against the above SDS-β-mercaptoethanol solution at 22 °C. Equal portions of supernatants and pellets were prepared for analysis by SDS-PAGE (39).

Densitometry

SDS gels were scanned for absorbance with a soft laser scanning densitometer (BioRad Instruments) using the tungsten lamp and a 520-nm filter. For each of the gels scanned, the photomultiplier gain was electronically set to optimum and the signal gain was adjusted so that peaks of interest were on scale. The zero gain signal was adjusted to a blank area on the gel. On each of the gels scanned, known bands corresponding to known milligram quantities of bovine serum albumin, Tm, and actin were also scanned in order to construct standard plots of micrograms of standard proteins applied on each gel versus the weight of each peak. These standard plots were used to calculate the relative molar ratios of actin and Tm in the samples analyzed by SDS-PAGE.

Actin-activated ATPase Assays

Actin-activated HMM Mg²⁺-ATPase activities were measured at 25 °C for 3.3 min in the ATPase buffer of 10 mM MOPS, pH 7.0, 85 mM KCl, 5.0 mM MgCl₂, 0.2 mM DTT, 0.005% NaN₃, 1 mM EGTA (for measurements in the absence of Ca²⁺). ATPase measurements in the presence of Ca²⁺ ions were performed by adding calcium (using a 0.1 M CaCl₂·2H₂O stock solution) to achieve a final free calcium concentration of 0.1 mM. The reaction was initiated by addition of ATP (using a 0.1 mM solution, adjusted to pH 7.0 with KOH) to 2 mM in a final reaction mixture volume of 2 ml. Termination of the reaction was achieved by addition of ice-cold trichloroacetic acid (50% stock) to a final 6% concentration. Stock solutions of actin (1.0 mg/ml), Tm (0.46 mg/ml), Tn (1.3 mg/ml), and HMM (4.5 mg/ml) were each in 10 mM MOPS, pH 7.0, 85 mM KCl, 0.2 mM DTT, 0.005% NaN₃. For the assays performed, actin, Tm, Tn, and HMM were mixed in a 7:1:1:1 molar ratio, respectively. Regulated actin filament protein complexes containing actin, Tm, and Tn were made by incubation of these proteins for 3 h at 22 °C with constant shaking. HMM was added last to the reaction mixture and all the protein mixtures were further incubated for 20 min at 25 °C before initiation of the ATPase reaction. ATPase activity was determined from the F, released (40). A single assay usually consisted of triplicate measurements.

Protein Concentrations

Protein concentrations were measured by the method of Lowry et al. (41) using bovine serum albumin as the color standard, or spectrophotometrically (on a Beckman Model 35 spectrophotometer) using the following extinction coefficients and molecular weights: troponin, E₅₀₀ = 0.45, 68,849 (42); tropomyosin, E₅₀₀ = 0.33, 65,000 (43); HMM, E₅₀₀ = 0.647, 350,000 (44). Unmodified actin concentrations were determined at 290 nm in order to minimize the contribution from ATP; extinction coefficients were E₅₀₀ = 0.617 for G-actin and E₅₀₀ = 0.638 for F-actin (45). The molecular weight of actin used was 43,000.

RESULTS

Modification of a Single Lysine in Actin by 2,4-Pentanedione

Spectrophotometric and Amino Acid Analyses—The reagent 2,4-pentanedione has been previously shown to react with the ε-amine group of lysine residues in proteins (27, 46-48) under conditions of low ionic strength and neutral pH to form an enamine product of \( \lambda_{max} \) at 312 nm (27):

\[
\text{CH}_2-\text{C}-\text{CH}_2-\text{C}-\text{CH}_2+\text{NH}_2-\text{lysine} \rightarrow \text{CH}_2-\text{C}-\text{CH}_2-\text{C}-\text{CH}_2+\text{H}_2\text{O}
\]

\[ 2,4\text{-pentanedione} \]

\[ \epsilon = 2.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1} \]

In addition, it can interact with the guanidine group of arginine residues at high ionic strength and at pH 9.0 to produce a pyrimidine product of \( \lambda_{max} = 500 \text{ nm} \) (27):

\[
\text{NH}_2\text{I}
\]

2,4-Pentanedione + NH₂-C-NH-Arg—

\[
\text{NH}_2\text{I}
\]

2,4-Pentanedione + NH₂-C-NH-Arg—

\[
\text{CH}_3
\]

\[
\text{CH}_3
\]

\[
\text{CH}_3
\]

\[
\text{NH}_2\text{Arg}
\]

\[
\text{pyrimidine}
\]
which are favorable for the modification of \( \epsilon \)-amino groups of lysine residues in proteins (27). Spectrophotometric UV analysis of the 2,4-pentanedione-treated G-actin (Fig. 1), in fact, shows an absorbance maximum at 312 nm, characteristic of enamine formation. This is further confirmed following treatment of the modified actin with hydroxylamine (at 15:1 molar ratio of \( \text{NH}_2\text{OH} \cdot \text{HCl} \) to actin lysines at pH 6.5), after which the enamine was converted back to an amine as evidenced (Fig. 1) by the loss of the 312-nm enamine absorbance (27). If the product had been a pyrimidine (due to arginine modification), it would be stable to hydrolysis upon hydroxylamine treatment and therefore an absorbance maximum at 300 nm (27), characteristic of a pyrimidine absorbance, would still be observed. Such a product did not exist since the spectra of the modified actin and control actin after hydroxylamine treatment were the same (Fig. 1). Based upon the above results and using a molar extinction coefficient of \( 2.1 \times 10^4 \) M\(^{-1}\) cm\(^{-1}\) for an enamine at \( \lambda_{\text{max}} \) of 312 nm (27), the number of lysine residues modified was determined to be 0.92 ± 0.12 mol/mol of actin. This result was the average of 12 experimental measurements.

The accuracy of the UV analysis for the extent of arginine modifications was also confirmed by acid hydrolysis and amino acid analysis of the modified and control actin. Under conditions of acid hydrolysis used for the modified and control actin (see "Experimental Procedures"), any pyrimidine present in the modified actin should convert into an ornithine, which elutes with the lysine peak in the amino acid analyzer (27). An increase in the area of the lysine peak, or a decrease in the area of the arginine peak with increasing extent of arginine modification, could be quantified. Results of several such analyses are shown in Table I. These results indicate that even at longer reaction times (e.g. for 24 h), where the extent of enamine formation was still the same (i.e. 1 mol of enamine formed/mol of actin as determined spectrophotometrically), there was no modification of arginine residues in G-actin, since the ratio of total lysine to arginine in control and modified G-actin samples remained essentially constant. No other differences in amino acid content were observed between native and modified G-actin.

Spectrophotometric measurements were also performed on F-actin after treatment with 2,4-pentanedione, and the results show that one lysine residue reacted with 2,4-pentanedione and that the absorbance maximum at 312 nm, due to enamine formation, was abolished (data not shown) after hydroxylamine treatment of the modified protein. In addition, amino acid analysis of control and modified F-actin (Table I) showed that the total moles of Lys/total moles of Arg ratio was essentially the same in control and modified F-actin samples, suggesting that no arginine residues were modified upon treatment of F-actin with 2,4-pentanedione for 5 h in the F-buffer.

Identification of the Single Lysine As Lys-237 in G-Actin—The above results were further confirmed by using a radiolabeled 2,4-pentanedione as the modifying reagent. For this purpose \([1-\text{\textsuperscript{14}C}]2,4\)-pentanedione was synthesized by the reaction of acetone with \([1-\text{\textsuperscript{14}C}]\)acetic anhydride in the presence of boron trifluoride as described by Doorenbos et al. (33). The Fourier transform NMR spectra of the synthesized \([1-\text{\textsuperscript{14}C}]2,4\)-pentanedione was identical to that of the commercially available unradioabeled 2,4-pentanedione (spectra not shown). The modification conditions using the radiolabeled 2,4-pentanedione were exactly the same as those employing the unlabeled reagent except that G-actin after modification was dialyzed against a pH 8.0 buffer (5 mM sodium bicarbonate, 0.2 mM ATP, 0.2 mM DTT) and not against the pH 7.0 G buffer, since the enamine product is relatively more stable at alkaline pH (27), and therefore favorable in the process of isolation of the peptide(s) carrying the radiolabeled product. The actin modified by \([1-\text{\textsuperscript{14}C}]2,4\)-pentanedione was found to have 0.92 lysine residue modified/mol of actin as determined spectrophotometrically (27), and radiochemical analysis indicated that 0.85 mol of \([1-\text{\textsuperscript{14}C}]2,4\)-pentanedione/mol of actin had been incorporated. Electrophoresis at pH 9.0 of the proteolytic digest of this labeled actin (Fig. 2) showed that a single radioactive band with an acidic mobility was present in addition to some radioactivity which remained at the origin and which was probably associated with incompletely digested

\begin{table}
\centering
\caption{Extent of modification of arginine residues in 2,4-pentanedione-treated actin.}
\begin{tabular}{|c|c|c|}
\hline
Experiment No. & Control & Modified \\
\hline
1 & 1.25 & 1.15 \\
2 & 1.15 & 1.18 \\
3 & 1.20 & 1.16 \\
4 & 1.14 & 1.17 \\
5 & 1.13 & 1.13 \\
6 & 1.23 & 1.16 \\
\hline
\end{tabular}
\end{table}

\*The ratios of lysine to arginine residues represents the ratio of the total number of moles of each of these amino acids as determined experimentally by amino acid analysis. Calculations of amino acid composition of each actin sample were determined in reference to the leucine content in each sample. The molar ratio of lysine to arginine residues in actin as published (15) is 1.05.

\*Modification with 2,4-pentanedione, was performed on F-actin for 5 h at 1.0 mg/ml in F buffer, as described under "Experimental Procedures."

\*Analysis performed on 1 mg of actin samples isolated from modified (for 10 h at pH 7.0) and control actin-Tm complexes.
protein. Further purification of the acidic radiolabeled peptide was achieved by descending paper chromatography (performed at alkaline pH in order to prevent enamine breakdown), and the single radioactive band \((R_f = 0.23)\) from the chromatogram was subjected to a further pH 9.0 electrophoresis in order to remove contaminants that interfere with the acid hydrolysis procedure (49).

Amino acid analysis of the peptide revealed that it had the following composition: Ser (0.8), Glx (1.1), Tyr (0.8), and Lys (1.0). The peptide, which was recovered in an overall yield of 8% from the original 1.28 \(\mu\)mol of actin digested, had a specific activity of 31.2 \(\times 10^6\) cpm/mol which is indicative of a single equivalent of 2,4-pentanediol incorporated/mol of peptide based on the specific activity of the radiolabeled 2,4-pentanediol used in the experiment.

After treatment at pH 3.5 to degrade the enamine in the peptide (27), N-terminal analysis of the peptide revealed Lys as the N-terminal residue, and electrophoresis at pH 6.5 indicated that the peptide was neutral. These results, together with amino acid analysis data and the known specificities of the proteolytic enzymes used for digestion, permitted an unambiguous identification of the peptide as the actin amino acid sequence for residues 237–240, so that the sequence of the labeled peptide must be Lys-Ser-Tyr-Glu and Lys-237 must be the single actin lysine residue modified by 2,4-pentanediol.

**Effects of G-Actin Modification on Its Structure**

Analysis of possible structural changes in the overall helical organization caused by treatment of G-actin with 2,4-pentanediol was performed using far-ultraviolet circular dichroism spectroscopy. The mean residue ellipticity values of the modified G-actin over the far-UV wavelength range of 210–250 nm were essentially the same as those for the control G-actin. In particular using \([\theta]_{221-225}\) values for comparison of actin structure (32), actin modified by 2,4-pentanediol had a negative ellipticity value almost identical \((-9583)\) to that of control G-actin \((-9570)\) (Fig. 3). These results indicate that the modification of a single lysine residue in G-actin with 2,4-pentanediol under the conditions specified under “Experimental Procedures” did not cause any alteration in the overall helical organization of the actin molecule. In addition, the modification had no significant effect on actin monomer-monomer association to form a polymeric F-actin upon dialysis of modified G-actin against the C buffer (see “Experimental Procedures”). This is confirmed by the results presented in Table II in which no significant variation in the reduced viscosities \(\left(\eta_m\right)\) of control and modified actin in the F-buffer was observed.

**Effects of Actin Modification on Its Biological Activities**

**Actin-Tm Interaction—Centrifugation, Electrophoresis, and Quantitative Densitometry of the Binding of Modified Actin to Tm at Physiological Ionic Strength**

The reduced viscosity \(\left(\eta_m\right)\) was calculated for control and modified actin samples (1.0 mg/ml) after overnight dialysis (in the cold) against the C buffer: 10 mM MOPS, pH 7.0, 100 mM KCl, 0.2 mM DTT, 0.2 mM ATP, 0.005% NaN\(_3\), \(\mu = 0.12\). Viscosity measurements were performed at 35°C, with the sample flow time measured separately for three different actin preparations, as described under “Experimental Procedures.”

| Sample            | \(\eta_m\) |
|--------------------|------------|
| Control actin      | 1.315      |
| Modified actin     | 1.294      |

**Table II**

**Effect of Lys-237 Modification on the Polymerizability of Actin**

The effects of actin modification on the polymerizability of actin were determined using the polymerization assay described under “Experimental Procedures.”

**Actin-Tm Interaction**

- Centrifugation, electrophoresis, and quantitative densitometry of the binding of modified actin to Tm at physiological ionic strength in the pH 7.0 C buffer \(\mu = 0.12\) in the absence of Ca\(^{2+}\) and Mg\(^{2+}\) demonstrated (Fig. 4) that the modified actin did not bind to Tm since no Tm was observed on the SDS gel with the modified actin pellet, and that all the unbound Tm appeared in the supernatant.

The degree of binding of control actin to Tm, under the same conditions, was not significantly affected. In fact, the number of moles of Tm bound per mole of control actin, 0.138 (Table III), compared closely to the stoichiometric degree of 0.143 as reported in myofibrils (50). The loss of ability of modified actin to bind to Tm was reversed after hydroxylamine treatment of the modified protein as shown in Fig. 5. F-actin was directly treated with 2,4-pentanediol for a short period of time \((5\ h)\), at \(\mu = 0.12\), and it also lost the ability to bind to Tm (which was also reversed by hydroxylamine treatment) and the modification had no effect on the polymeric state of the protein (results not shown).

To determine whether Lys-237 was directly involved in actin-Tm interaction, modification experiments were performed on an F-actin-Tm complex in the absence of Ca\(^{2+}\) and Mg\(^{2+}\) at pH 7.0 (C buffer) and pH 8 for 10-h and 5-h reaction times, respectively. In each case, spectrophotometric measurements on actin isolated from the actin-Tm complex showed that 0.2–0.3 mol of enamine/mol of actin was incorporated. This is compared to a value of 0.7–0.8 mol of enamine/mol of a modified F-actin that was treated with 2,4-pentanediol in the absence of Tm and subjected to the
Effects of Ca\(^{2+}\) and Mg\(^{2+}\) Ions on Modified Actin Interaction with Tm—The effects of divalent cations on the interaction between modified actin and Tm were investigated under the ionic strength conditions of the C buffer (pH 7.0) in the presence of 5 mM Mg\(^{2+}\) and in the presence (pCa = 4.0) and absence of Ca\(^{2+}\). Binding of Tm to modified actin was restored by Mg\(^{2+}\) as determined by SDS-PAGE of the supernatants and pellets after centrifugation of the protein mixtures (results not shown). However, under such conditions, the number of moles of Tm bound/mol of modified actin was about 25% less than that of Tm bound/mol of control actin (Table III). When both Ca\(^{2+}\) and Mg\(^{2+}\) were present, the ability of the modified actin to bind Tm was enhanced, and the number of moles of Tm bound/mol of modified actin represented about 91% of that bound to control actin (Table III).

Presence of Tn—Modified actin was mixed with Tm in the presence of Tn at an actin:Tm:Tn molar ratio of 7:1:1 in C buffer, p\(
\mu\) = 0.12, pH 7.0. In the absence of any divalent cation, it was observed that Tn restored Tm binding to the modified actin as demonstrated by SDS-PAGE (results not shown). The degree of Tm binding/mol of modified actin (0.135) was equivalent to that for control actin (0.138) as quantified from densitometric scans of their SDS-PAGE. The addition of Mg\(^{2+}\) in the presence or absence of Ca\(^{2+}\) had no further effect on Tm binding (Table III).

**Actin Activation of HMM-Mg\(^{2+}\)-ATPase Activity**

The effects of actin modification on the ability of actin to activate the HMM-Mg\(^{2+}\)-ATPase activity was investigated in the C buffer and at an actin:HMM molar ratio of 7:1. It was determined that the specific activity (actin-HMM-Mg\(^{2+}\)-ATPase/mg of HMM/min) was essentially the same whether modified or control actin was the ATPase activator.

The ATPase activities were also investigated in the presence of Tn and in the presence of Tm and Tn. Under stoichiometric conditions in which 1 mol of Tm binds to nearly 7 mol of control actin (i.e. at 0.140 mol of Tm bound/mol of actin), a 55% inhibition of actin-activated HMM-ATPase activity was observed (Fig. 6) which is consistent with earlier data produced under nearly equivalent ionic strength conditions (51) but at an actin:Tm molar ratio of 2:1 and an actin:HMM molar ratio of 14.6:1. However, in the same state and under conditions in which 0.102 mol of Tm was bound per mol of modified actin, there was no significant inhibition of the ATPase activity observed (97% activation relative to control actin in the absence of Tm) (Fig. 6).

In the case of regulated unmodified actin (i.e. in the presence of Tm and Tn) in the absence of Ca\(^{2+}\), the ATPase

### Table III

| Experimental conditions | Control | Modified |
|-------------------------|---------|----------|
| mol Tm bound/mol actin  |         |          |
| 1. No Ca\(^{2+}\) or Mg\(^{2+}\) ions | 0.138   | 0.0      |
| 2. 5 mM Mg\(^{2+}\)  | 0.137   | 0.102    |
| 3. 0.1 mM Ca\(^{2+}\), 5 mM Mg\(^{2+}\) | 0.145   | 0.132    |
| 4. Tn (Ca\(^{2+}\), Mg\(^{2+}\)) | 0.138   | 0.135    |
| 5. Tn (5 mM Mg\(^{2+}\)) | 0.140   | 0.137    |
| 6. Tn (0.1 mM Ca\(^{2+}\), 5 mM Mg\(^{2+}\)) | 0.150   | 0.144    |

identical procedure used for the isolation of actin from a modified complex of actin-Tm. In the actin isolated from the modified complex, there were no arginine residues modified since the mole ratio of lysine to arginine residues was equivalent to that from a control actin from an unmodified complex of actin-Tm (Table I).
activity was further inhibited (80%). However, under similar conditions, the ATPase activity associated with regulated modified actin was further enhanced to levels (about 133%) above those produced in the absence of Tm and Tm-Tn (100%) (Fig. 6). On the other hand, Ca2+ addition to the above systems caused a relief of the inhibition of the ATPase activity associated with the regulated unmodified actin (reaching 100% activity), it slightly enhanced the ATPase activation by the regulated modified actin to nearly 12% higher (or 145% activity) than that observed in the absence of Ca2+ (Fig. 6).

**DISCUSSION**

Results from spectrophotometry, amino acid analysis, and radiolabeling studies indicate that approximately one equivalent of 2,4-pentanediene is covalently attached per mol of G-actin under the selected modification conditions described. Electrophoresis of the digest of radiolabeled modified actin revealed the presence of a single radioactive peptide in the digest. Analytical data for this peptide clearly indicate it to be residues 237–240 of the actin sequence involving Lys-237 as the modified lysine residue. The production of this peptide from the digest is consistent with the cleavage by *S. aureus* protease of peptide bonds between residues 236–237 (Glu-Lys) and 240–241 (Glu-Leu) in the actin amino acid sequence (15), by virtue of the enzyme specificity for peptide bonds of glutamyl residues (52). That the labeled lysine appears as an N-terminal residue in a tryptic fragment suggests that either the enamine derivative of lysine cannot occupy the active site of the trypsin molecule, or that a prior cleavage of the peptide bond between residues 236–237 renders the peptide bond between residues 237 and 238 (Lys-Ser) resistant to cleavage by trypsin. Since no evidence was found for the presence of radiolabeled modified Lys-237 attached to Glu-236 in other peptides in the digest, it would appear likely that trypsin is unable to bind the enamine derivative of the lysine side chain and that these modified residues are therefore completely resistant to tryptic digestion.

The labeled peptide was found to have an acidic mobility at pH 9.0. After degradation of the enamine group, the peptide had a neutral mobility at pH 6.5. These results indicate that the enamine derivative of the side chain of Lys-237 carries no net charge at pH 9.0 (giving the peptide an overall net charge of –1 at this pH) and that the unblocked peptide (Lys-Ser-Tyr-Glu) carries a net charge of zero at pH 6.5.

The polymeric F-actin modified with 2,4-pentanediene was compared to F-actin prepared from modified G-actin by dialysis against F buffer. In assays of the number of lysines modified per actin monomer, ability to interact with Tm, and polymer stability, the data suggest that the same lysine residue, i.e., Lys-237, is modified in either case. The Lys-237 microenvironment might therefore represent an accessible site on the actin monomer that is not involved in monomerymeronmer association. In fact, studies on the reactivities of lysine residues in both G- and F-actin (25) have demonstrated that most lysine residues, including Lys-237 remains unchanged upon G-to-F transformation.

The lysine modification seems to have no effect on (a) the secondary structure of modified G-actin (judged from CD analysis), (b) the maintenance of the polymeric state of modified actin, or (c) the stimulation of HMM-MgATPase activity by the modified actin. However, in the absence of Ca2+ and Mg2+, the modification did result in impaired Tm binding to actin, suggesting either that it disturbed the conformation of a site for the interaction with Tm around Lys-237 of actin or that it induced an overall conformational change in actin affecting a Tm-binding site more distant from Lys-237. Prior interaction of actin with Tm allowed only minimal modification of the actin, although no attempt was made to specifically determine that Lys-237 was the residue thus “protected.” In fact, only 0.2–0.3 mol of modified lysine was found per mol of actin isolated from an actin-Tm complex reacted with 2,4-pentanediene. This observation, compared to an average of 0.75 mol of modified lysine/mol of actin reacted while Tm-free, suggests that from 100% (no interacting Lys-237) to 67% (assuming the 0.2–0.3 mol of lysine interacting is solely Lys-237) protection of Lys-237 from modification was achieved by prior interaction of actin with Tm. This could result either from direct binding of Tm at the site on actin containing Lys-237 or from structural changes around Lys-237 induced by Tm binding elsewhere on actin.

The inability of modified actin to bind Tm (in the absence of Ca2+ and Mg2+, μ = 0.12) can be restored by the addition of either Tn, Mg2+, or Ca2+. The exact mechanism by which Tn and these cations may act to restore Tm binding cannot be clearly explained at this point. It is certain, however, that the restoration of Tm binding by modified actin with addition of divalent cations was not due to the presence of contaminating Tn.

The modification of actin at Lys-237 does alter the normal properties of actin-HMM-MgATPase activity observed in the presence of Tm alone or with Tn (Fig. 6). In fact, the ATPase activation by modified actin (with ~1 Tm bound/7 actins) was not inhibited under conditions in which nearly equivalent degrees of Tn binding to control actin caused significant inhibition of the ATPase activity. Even when Tn was added to the modified system (in the absence of Ca2+), no inhibition of ATPase could be observed. This suggests that the modification prevents the F-actin-Tm from assuming the

**Fig. 6.** The modified actin activation by HMM-Mg2+-ATPase activity in the presence of Tm and Tm-Tn and in the absence and presence of Ca2+. ATPase measurements were performed at 25 °C for 3.3 min in a final 2-ml assay volume of C buffer containing 10 mM MOPS, pH 7.0, 85 mM KCl, 5.0 mM MgCl2, 0.2 mM DTT, 0.005% NaN3, 1 mM EGTA (for measurements in the absence of Ca2+), and 0.1 mM free Ca2+. Actin, Tm, Tn, and HMM were mixed in a 7:1:1:1 molar ratio, respectively, as detailed under "Experimental Procedures." ATP was added to 2 mM to initiate the reaction. The reaction was terminated by adding trichloroacetic acid to 6% final concentration. The specific ATPase activity for each sample was determined from the P32 released (40) and was corrected for the specific activity contributed by HMM alone. Percentage of ATPase activity = specific activity of sample/specific activity of control actin without Tm or Tm-Tn × 100. The number of moles of Tm bound/mol of actin is enclosed in each bar representing the per cent ATPase of each sample. The Tm bound in each case was as determined earlier for each state (Table III).
blocking inhibitory position (as described in the “steric blocking” theory) or conformation (that may inhibit a kinetic step in the ATPase cycle as suggested by Chalovich and Eisenberg (11). On the other hand, the fact that Tn appears to activate the ATPase of the modified F-actin-Tm complex whether ATP concentrations (53). In short, using the modified and unmodified actin system, it is possible to observe three actin.

Ca++ is present or not suggests that this state may resemble or observation of each state may be dependent on the partic-

ular conditions (e.g. myosin head ratio to actin, temperature, or salt) which happen to be used for the ATPase measure-

ments.

These adds should be very useful for studying the inter-

action of HMM with actin in order to answer questions about the possible change in affinity of actin for HMM in these different states. They should also be useful for studying the different positions which Tm may assume in the modified F-actin-Tm versus the unmodified F-actin-Tm in three-dimen-

sional reconstructions of thin filaments. Another question which can be asked is whether this modification affects the Tn-Ca++ affinity in a regulated actin filament. We are cur-

rently pursuing these questions in our laboratory.

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