Cross-Linked Dimers with Nucleating Activity in Actin Prepared from Muscle Acetone Powder[†,‡]

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ABSTRACT: A covalently linked actin dimer is identified in solutions of actin prepared from an acetone powder from skeletal muscle. This actin dimer acts as an actin nucleating factor (ANF), decreasing the half-time for spontaneous actin polymerization. ANF reacts with antibodies to both the N- and C-terminal portions of actin on Western blots and migrates during reduced polyacrylamide gel electrophoresis like actin cross-linked with *N*,*N*′-*p*-phenylenebismaleimide. The origin of the cross-linked dimer appears to be related to the presence of carbonyl groups in purified actin. A large number of carbonyls (∼0.3/actin) are introduced into actin during the prolonged treatment with acetone in the preparation of the muscle acetone powder from which actin is extracted. Actin extracted from acetone powder prepared by a single acetone wash and actin prepared from bovine spleen, which is not washed with acetone, both contain fewer carbonyl groups (∼0.05 carbonyl/actin). ANF forms spontaneously in solutions of polymer actin containing 0.3 carbonyl/actin. We speculate that a reaction between a carbonyl on one actin polymer subunit and a lysine on a neighboring subunit is responsible for ANF formation. The presence of cross-linked actin dimers in commonly used skeletal muscle actin preparations could certainly affect studies of actin polymerization and, particularly, studies of the nucleation reaction. The physiological relevance of ANF is not clear, but given the large cellular concentration of actin, similar reactions yielding ANF could occur in vivo when increased levels of reactive oxygen species are present.

The eukaryotic protein actin is essential to cell shape and motility. Formation of long polymers of F-actin from monomeric G-actin occurs via a nucleation−elongation mechanism (*1*). The nucleation reaction is a third- or fourth-order rate-limiting reaction that results in filament ends onto which G-actin monomers can associate via a second-order elongation reaction. Typically, in studies of spontaneous actin polymerization in vitro, the filament end concentration is in the sub-nanomolar range, and even minute concentrations of agents that alter the filament end concentration can have significant effects on the actin polymerization rate.

Actin prepared from rabbit skeletal muscle acetone powder has been the standard for the vast majority of actin polymerization studies. It has been known for some time that small concentrations of contaminant proteins that can alter the kinetics of actin polymerization may be present in actin preparations. MacLean-Fletcher and Pollard (*2*) first addressed this issue when they noted a contaminant that altered the polymerization characteristics and length distribution of actin polymer solutions. Later, Casella et al. (*3*) showed that a protein called CapZ was responsible for these effects and that the preparation of actin free of CapZ requires multiple column purification steps (*4*).

In this report, we describe an actin nucleating factor (ANF) in actin solutions prepared from skeletal muscle acetone powder. ANF appears to be a covalently cross-linked actin dimer, similar to the synthetic upper dimer described by Millonig et al. (*5*). Our investigation reveals that the dimer most likely results from a reaction between carbonyl groups and lysine residues on adjacent actin subunits within a polymer. Carbonyl residues appear to be introduced during the prepartion of muscle acetone powder, and the modified actin copurifies with normal actin. Thus, actin solutions contain variable amounts of carbonyl-containing actin which, when polymerized, can spontaneously form covalently cross-linked actin dimers (ANF) leading to altered kinetics during subsequent cycles of actin polymerization.

The dimers present in actin prepared from muscle acetone powder appear to be artifacts of the preparation and may be important only for their effects on laboratory studies of actin polymerization. However, the presence of carbonyl residues in actin has been reported to occur in vivo during oxidative stress (*6*), and thus, in vivo generation of ANF is possible. This could have significant effects on the stability and dynamics of the actin cytoskeleton.

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MATERIALS AND METHODS

Acetone Powder Preparation. In the following procedure, the volumes given are per 100 g of muscle. Back and leg muscle from a freshly sacrificed rabbit was immediately embedded in ice. The visible fat was trimmed, and the muscle was ground in a commercial meat grinder (1−2 mm holes) within 45 min. Myosin was extracted with 300 mL of 0.3 M $KHPO_4$ and 0.3 M KCl (pH 6.5) for 10 min with stirring at 5 °C. This solution was diluted with 1200 mL of water and filtered through cheesecloth. The muscle residue was suspended in 500 mL of 47.6 mM $NaHCO_3$ and 0.1 mM $CaCl_2$ and the mixture stirred for 30 min at room temperature. The muscle residue was then collected by filtration through cheesecloth and suspended in 100 mL of 10 mM $NaHCO_3$, 10 mM Na_2CO_3, and 0.1 mM $CaCl_2$ at 5 °C for 10 min, after which 1 L of water was added and the solution quickly filtered through cheesecloth. The residue was next suspended in 200 mL of cold (5 °C) acetone, stirred for 30 min at room temperature, and collected by filtration through cheesecloth, and the residue was squeezed thoroughly to remove as much acetone as possible. This process was repeated with another 200 mL of cold acetone, and the resulting residue was homogenized with a stainless steel blade in small batches in an ice-cold Sorvall Omni-Mixer for 5−7 s. The residue was then washed three more times, each wash with 200 mL of cold acetone. The final residue was spread on, and covered with, filter paper and dried overnight in a fume hood. The next day, the dried muscle residue was homogenized in the Sorvall Omni-Mixer for 7−10 s, packed into tubes, and stored at −20 °C.

Skeletal Muscle Actin Preparation. Extraction of actin from rabbit skeletal muscle acetone powder was typically accomplished by a modification of the method of Spudich and Watt (*7*). Alterations in the procedure were found to have no significant effects on the amount of ANF in preparations. Typically, 5 g of acetone powder was extracted for 20 min at 4 °C with 100 mL of 2 mM Tris-HCl,[1] 0.2 mM ATP, 0.1 mM $CaCl_2$, and 0.5 mM DTT (pH 8.0). The mixture was centrifuged at 10000*g* for 20 min, and the supernatant was further clarified by centrifugation at 190000*g* for 30 min. The supernatant (∼60 mL) was converted to MgATP−actin by a 6 min incubation with 0.1 mM $MgCl_2$ and 0.5 mM EGTA, then polymerized by addition of KCl to a final concentration of 100 mM and $MgCl_2$ to a final concentration of 2 mM, and kept at 5 °C overnight. The next day, KCl was added to a final concentration of 0.8 M and the solution stirred slowly for 1.5 h at 5 °C. The solution was centrifuged at 190000*g* for 2 h, and the F-actin pellets were homogenized in 50 mL of 2 mM Tris-HCl, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM EGTA, and 1.5 mM NaN_3 (pH 8.0) at 5 °C. The actin solution was then centrifuged at 190000*g* for 2 h, and the pellets of F-actin were covered with a small volume of 2 mM Tris-HCl, 1 mM ATP, 0.2 mM $CaCl_2$, and 1.5 mM NaN_3 (pH 8.0) at 5 °C. The pellets were allowed to soften for 2 h with occasional agitation. The softened pellets were then homogenized in 10 mL of 2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 1.5 mM NaN_3 (pH 8.0) at 5 °C and dialyzed at 5 °C for 48 h against 500 mL of this buffer, with a buffer change every 12 h. If birefringence was noted after this time (indicating the presence of polymer), the actin was diluted to a final concentration of 100 μM, sonicated for two 15 s periods on ice with the micro tip of a Cole Parmer ultrasonic homogenizer, and dialyzed at 5 °C for an additional 24 h. The actin solution was then clarified by centrifugation for 3 h at 190000*g*, and the supernatant (∼10 mL) was filtered through a 0.22 μm filter. The actin was further purified by gel filtration on a 2.5 cm × 100 cm column of Sephacryl S300 HR equilibrated with 2 mM Tris-HCl, 0.2 mM ATP, 0.02 mM $CaCl_2$, and 0.01% NaN_3 (pH 7.5) at 5 °C (buffer G). Preparations in which HEPES buffer at pH 7−7.5 was substituted for Tris-HCl exhibited the same characteristics, and the presence of 0.5 mM DTT throughout the procedure had no detectable effect on the final preparation.

Non-Muscle Actin Preparation. Non-muscle actin was purified from bovine spleen using poly-L-proline affinity chromatography (*8*). All procedures were carried out at 5 °C. The spleen was obtained from Pel-Freez Biologicals (Rogers, AR) as 1 cm cubes which had been frozen on dry ice immediately after removal from the animal. The spleen tissue (90 g) was homogenized, and the homogenate was clarified by centrifugation at 16000*g* for 20 min. The supernatant was further clarified by centrifugation for 30 min at 145000*g* and applied to a 5 cm × 5 cm polyproline column, and the column was washed as described by Lindberg et al. (*8*). Actin was eluted from the washed column with 0.5 M KI according to the method described by Goldschmidt-Clermont et al. (*9*). The actin peak was detected by tryptophan fluorescence with excitation and emission wavelengths of 300 and 335 nm, respectively. The pooled fractions (200 mL) were immediately dialyzed at 5 °C against two 2 L changes of 2 mM Tris-HCl, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, and 0.5 mM DTT (pH 7.5) and centrifuged at 190000*g* for 2 h so the polymerized actin could be collected. The pellets were homogenized and depolymerized by dialysis against buffer G containing 0.2 mM DTT.

Preparation of PBM-Cross-Linked Actin Dimers and Trimers. PBM-cross-linked dimers were prepared by the method described by Mockrin and Korn (*10*), and trimers by the method of Gilbert and Frieden (*11*). These preparations were purified and characterized by gel filtration chromatography at 5 °C in a 2.5 cm × 100 cm Sephacryl S300 HR column and SDS−PAGE analysis of the column fractions.

Sephacryl S300 HR Gel Filtration. A 2.5 cm × 100 cm column was packed according to the manufacturer's instructions and typically run at 5 °C at a flow rate of 1 mL/min. The column was calibrated by application of a mixture of actin monomer and PBM-cross-linked dimer, trimer, and oligomers, and the eluted proteins were analyzed by SDS−PAGE on a 7.5% gel. Electrophoresis of the column fractions showed two types of dimers with apparent molecular masses of 84 and 115 kDa, and a series of trimer bands with apparent molecular masses of 160−180 kDa, as well as higher-order

[1] Abbreviations: MgATP−actin, monomeric (G) actin which contains bound MgATP; Cc, critical actin concentration; DTT, dithiothreitol; PBM, *N*,*N*′-*p*-phenylenebismaleimide; DNPH, 2,4-dinitrophenylhydrazine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(morpholino)-propanesulfonic acid; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; PVDF, polyvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GPDH, glycerol-3-phosphate dehydrogenase; IEF, isoelectric focusing.

oligomers that appeared in the void volume.

Actin Polymerization Experiments. Before polymerization, actin was converted to MgATP−actin by a 6 min incubation with 0.1 mM $MgCl_2$ and 0.1 mM EGTA (*12*). Polymerization of the sample was initiated by addition of concentrated KCl and $MgCl_2$ solutions to yield final concentrations of 100 mM KCl and 2 mM $MgCl_2$. For the experiments described herein, samples had a small (<10%) amount of pyrene-labeled monomeric actin added, and polymerization was monitored by fluorescence with excitation and emission wavelengths of 366 and 386 nm, respectively. Experiments without added pyrene, but using the intrinsic tryptophan fluorescence to monitor polymerization (*13*), yielded identical results. The pyrene fluorescence method was preferable because the signal-to-noise ratio upon polymerization was better than that for tryptophan fluorescence measurements.

ANF Activity Assay of Sephacryl S300 HR Column Fractions. An aliquot of each fraction was added to buffer G, and the final concentration of actin in each sample was adjusted to 0.5 μM using a fraction from the trailing portion of the actin peak (as will be shown below, this fraction contains minimal ANF activity). A low concentration (10−50 nM) of pyrene-labeled actin was included in each sample, and the sample was polymerized as described above. The rate constant for polymerization

$$k_{obs} = 0.693/(t_{1/2} \text{ for polymerization})$$

and the relative ANF activity

$$t_{1/2}^{\text{control}}/t_{1/2}^{\text{sample}}$$

were determined for each sample, where the control sample contained actin from the trailing peak only.

DEAE Chromatography. Either DE52 from Whatman International Ltd. (Maidstone, England) or DEAE-Sepharose FF from Pharmacia Biotech (Uppsala, Sweden) was used to separate ANF and PBM dimer from most of the monomeric actin. The column media were prepared according to the manufacturer's instructions and equilibrated with buffer G at 5 °C, containing 0.5 mM DTT, until the ATP concentration of the effluent was constant, indicating that the column matrix was saturated with ATP. Pooled fractions containing ANF or PBM-cross-linked dimers from Sephacryl S300 HR gel filtration were applied to a 0.9 cm × 30 cm column. The column was eluted with either a 400 mL linear gradient of buffer G containing 0 to 500 mM KCl or a step gradient consisting of buffer G containing 0.125, 0.2, and 0.3 M KCl.

SDS−PAGE was accomplished on a Mini-Protean apparatus from Bio-Rad (Hercules, CA), following the protocol of Lammeli (*14*). All electrophoresis samples were heated in the presence of DTT or 2-mercaptoethanol to ensure reduction of disulfide bonds. Molecular mass markers were applied to one lane of each gel for calculation of the apparent molecular masses. Actin samples were concentrated 30−100-fold by polymerizing the actin and collecting the polymer by centrifugation at 228000*g* for 30 min. The pellets were dissolved in Lammeli sample buffer (*14*) and heated to 100 °C for 5 min before being applied to the gel.

Isoelectric focusing (IEF) was accomplished in the presence of 8 M urea using the mini-slab gel format described by Bollag and Edelstein (*15*).

Western Blotting. Proteins were transferred from polyacrylamide gels to PVDF membrane by the method of Towbin (*16*). Monoclonal anti-α-sarcomeric actin (clone 5C5) and monoclonal anti-actin (clone AC-40), and anti-DNP antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). Appropriate alkaline phosphatase-coupled secondary antibodies were used, and the antibody reaction was visualized using an Alkaline Phosphate Conjugate Substrate Kit from Bio-Rad.

Molecular Mass Determination by Ultracentrifugation. Ultracentrifugation was accomplished in a Beckman Airfuge rotor by the method developed by Bothwell et al. (*17*). In addition to protein, each sample (0.183 mL) contained 80 kDa dextran (5 mg/mL) for stabilization during sample manipulation. Internal standards with known molecular masses were included in each centrifuge run, and the samples were centrifuged at top speed for 6 h. After centrifugation, a 40 μL aliquot was taken from the top of each tube, assayed, and referenced to the original sample. Intrinsic fluorescence was used to assay the protein concentration of monomer actin (43 kDa), gelsolin (84 kDa), and glycerol-3-phosphate dehydrogenase (145 kDa). Samples of ANF, PBM-cross-linked actin dimer, and PBM-cross-linked actin trimer contained low concentrations of protein (and in the case of ANF, also contained monomer actin) and were therefore assayed by measuring the relative nucleating activities before and after centrifugation.

Carbonyl Content Determination. Quantitative determination of the carbonyl content of actin was achieved by the method of Levine et al. (*18*). Typically, 0.5−1 mg of actin was precipitated by addition of TCA to a final concentration of 10% and collected by centrifugation at 13000*g* for 10 min. The resultant pellet was dispersed in 0.5 mL of 10 mM DNPH in 10 mM HCl and reacted for 1 h at 22 °C with occasional mixing, after which 0.5 mL of 20% TCA was added and the precipitated actin collected by centrifugation for 10 min at 13000*g*. The pellet was washed three times by dispersal in 0.5 mL of 1:1 ethyl acetate/ethanol (v:v) followed by centrifugation to collect the pellet. The final pellet was dissolved (15 min at 37 °C) in 0.6 mL of 6 M guanidine and 20 mM potassium phosphate, brought to pH 2.3 with TFA. The carbonyl concentration was calculated from the optical density at 370 nm, using an extinction coefficient of 22 000 M^{-1} cm^{-1}.

For qualitative identification of carbonyls in actin samples subjected to SDS−PAGE, we used the method of Schacter et al. (*19*) in which a sample is reacted with DNPH prior to SDS−PAGE, and detected by Western blotting using anti-DNP antibody. This procedure was modified for the detection of carbonyls on actin samples subjected to IEF. To avert any modifications in isoelectric point due to the reaction with DNPH, the samples were first subjected to IEF and then reacted with DNPH. After the IEF was complete, the 0.75 mm thick mini-slab gel was washed for 5 min with 10% TCA followed by three 5 min water washes. Enough freshly prepared 10 mM DNPH in 2 N HCl to completely cover the gel was added, and the gel was gently agitated for 15 min, washed with water until visibly clear of yellow color (typically five washes), and incubated for 5 min in transfer buffer. Western blot detection of DNP was performed as described above.

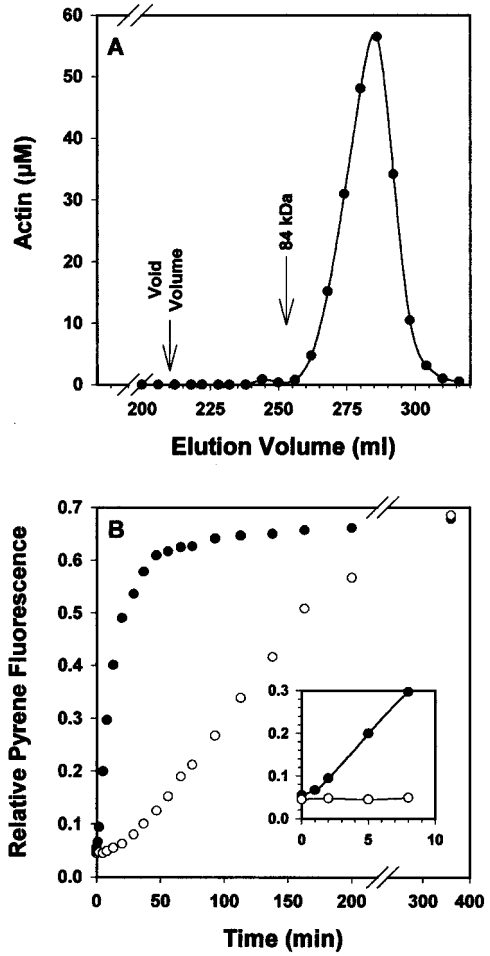

FIGURE 1: Gel filtration of actin preparations. (A) Elution profile for gel filtration of actin on Sephacryl S300 HR. A 10 mL sample of 100 μM actin was loaded on a 2.5 cm × 100 cm column packed with Sephacryl S300 HR. The column was eluted at a flow rate of 1 mL/min, and 6 mL fractions were collected. (B) Leading edge fractions (elution volume of 250−268 mL) were pooled to yield a 5 μM actin solution approximately equal in concentration to a trailing fraction (elution volume of 295−301 mL). Both samples were diluted to a final concentration of 0.5 μM and polymerized as described in Materials and Methods. Black symbols represent data for the leading edge fraction and white symbols for the trailing edge fraction. The inset shows that the lag phase early in the polymerization reaction was much shorter for the leading edge fraction than for the trailing edge fraction.

Mass Spectrometry. Electrospray ionization (ESI) mass spectra were obtained with a Finnigan TSQ 700 triple-quadrupole instrument at the Biological Mass Spectrometry Laboratory, Wadsworth Center for Laboratories and Research, Albany, NY. Actin solutions, 25 μM in 25% acetonitrile and 0.1% formic acid, were infused at a rate of 400 nL/min via a 20 μm inside diameter fused silica capillary. Multiply charged ion data were deconvoluted to obtain protein molecular masses.

RESULTS

Polymerization of Fractions from Actin Gel Filtration. In studies of the actin nucleation reaction over the past several years, we noted a significant degree of variability in nucleation rates among different actin preparations. Figure 1A shows the elution profile of actin from a Sephacryl S300 HR column. By testing different fractions from the final column purification step in the preparative procedure, we found an increased nucleating activity in the leading edge (Figure 1A, elution volume of 250−268 mL) of the actin peak (*2*). The amount of this nucleating activity varied from preparation to preparation. The barbed end capping protein, CapZ, is known to be present in the leading edge of the actin peak. However, at actin concentrations below the critical concentration for the pointed end of the polymer, material from the leading edge of the peak increased the nucleation rate, whereas under these conditions, CapZ would be expected to decrease the polymerization rate.[2] Thus, this nucleating activity was not due to CapZ.

Figure 1B compares the time course for polymerization of an actin sample eluted at 250 mL (leading fraction) to one eluting at 298 mL (trailing fraction). The actin concentrations of the two samples were equal (0.5 μM), and both actin samples reached the same final plateau, indicating that the same amount of polymer was formed in each assay, despite the dramatically different rates of polymer formation. Both polymerization curves have the sigmoid shape characteristic of a nucleation−elongation reaction, but the polymerization rate of the sample from the leading edge fraction is much faster. Since the actin concentration in this experiment is below the critical concentration for the pointed end of the actin filament, the result could be accounted for by hypothesizing a barbed end capping protein in the trailing column fraction and/or a nucleating activity in the leading column fraction. The inset of Figure 1B shows that the (nucleation) lag phase activity is much reduced for the leading edge fraction, strongly suggesting the presence of nucleating activity in this fraction. Other experiments (not shown) demonstrated that heating an aliquot from the leading edge of the actin peak for several minutes at 60 °C before adding it to the assay solution destroyed its nucleating activity. We thus postulated that the increased nucleating activity in the leading fractions is due to an actin nucleating factor (ANF) which is able to overcome the well-documented inhibiting (capping) effects of CapZ.

Characterization of Actin Nucleating Factor. To further characterize ANF, we pooled and polymerized gel filtration fractions containing ANF activity, concentrated the F-actin by centrifugation, and depolymerized the sample by dialysis. This actin was then reapplied to a Sephacryl S300 HR

[2] ANF and CapZ have opposite effects on actin polymerization at actin concentrations below the Cc for the pointed end of the actin filament. At higher actin concentrations, the two proteins could both increase the polymerization rate: ANF by increasing the number of barbed and pointed filament ends and CapZ by increasing the number of pointed filament ends. At concentrations below the Cc for the pointed end, net polymerization occurs only on barbed ends, which may be blocked by CapZ (*18*). We demonstrated the antagonist effects of the two proteins at actin concentrations below the pointed end Cc in a set of experiments not included here. Using anti-CapZ antibody kindly provided by J. Cooper and D. Shafer, we removed a portion of the CapZ from the actin samples and observed an increase in the ANF activity in the sample. Thus, the measured ANF activity does not necessarily reflect the true ANF content of a sample, but depends on the relative concentrations of CapZ and ANF. We found that CapZ can be assayed independently using an assay identical to that described for ANF (see Materials and Methods). Small (0.1 mL/2 mL of assay solution) aliquots are incubated overnight with 2 mM EDTA at room temperature before addition to the assay solution containing actin. This process denatures the ANF (and actin) in the aliquot, leaving only CapZ. The effect of capping on the actin polymerization rate was a very sensitive measure of the relative concentration of CapZ in each fraction, and was comparable in accuracy to CapZ determinations by dot blot analysis.

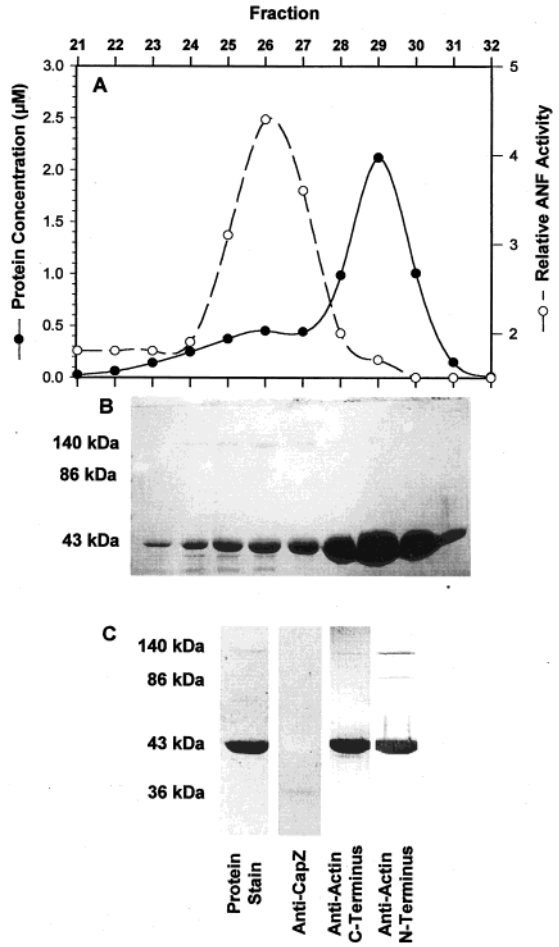

FIGURE 2: Identification of a 140 kDa protein band in ANF-containing fractions. (A) ANF-containing fractions from Sephacryl S300 HR gel filtration were pooled and polymerized. The F-actin was collected by centrifugation at 190000*g* for 90 min, and the resulting pellets were homogenized in buffer G, dialyzed for 48 h, and rechromatographed on the S300 HR gel filtration column. The protein concentration (●) and the relative ANF activity (○) of each fraction were determined as described in Materials and Methods. (B) The Coomassie brilliant blue-stained 10% SDS−PAGE of fractions (referenced to fraction numbers in the gel filtration chromatogram in panel A). Each sample was concentrated 30-fold and dissolved in Lammeli buffer (*14*) before electrophoresis. (C) A gel filtration fraction with peak ANF activity (similar to fraction 26 in panel A) was assessed by Western blot analysis of an SDS−PAGE gel (Sypro Ruby Red stain, lane 1) using antibodies against CapZ (lane 2), the C-terminal peptide of skeletal muscle actin (lane 3), and an N-terminal epitope of skeletal muscle actin (lane 4). The PVDF membrane was stripped for 30 min with 62.5 mM Tris-HCl, 2% SDS, and 100 mM β-mercaptoethanol (pH 6.8) at 50 °C between probes for the different antibodies.

column that had been calibrated with molecular mass standards. The protein elution pattern is shown in Figure 2A (●) along with the nucleating activity of each fraction (○). The peak ANF activity elutes in the leading edge of the protein peak at an elution volume equivalent to that of an 84 kDa protein, while the main protein peak elutes at 42 kDa. After concentration by polymerization and sedimentation, each fraction was subjected to 10% reduced SDS−PAGE. Figure 2B shows that the ANF fractions contained a protein band with a molecular mass estimated to be 140 kDa (by comparison to molecular mass standards), which was not present in fractions from the trailing edge of the protein peak. The proteolytic fragments below the actin band in Figure 2B were variable from preparation to preparation and did not correlate with nucleating activity. Lane 1 of Figure 2C contains an ANF-containing peak fraction from another preparation. In this case, proteolysis was eliminated by including 1 mM PMSF, 20 μg/mL aprotinin, and 20 μg/mL cystatin throughout the preparation. Lane 2 of Figure 2C shows that the peak fraction of ANF activity contains CapZ, as identified by Western blot analysis. Lanes 3 and 4 of Figure 2C show that the 140 kDa band is reactive to antibodies specific for the N-terminus and C-terminus of actin, respectively.

ANF Is a Cross-Linked Actin Dimer. We initially thought that ANF might be an actin-binding protein from muscle that copurified with actin. However, several experiments suggested that ANF was similar to actin itself. Removing ATP or divalent cation from a sample containing ANF activity destroyed its polymerization enhancing ability. In a series of experiments (not shown), the rate of ANF inactivation at low divalent cation concentrations exhibited a dependence on ATP concentration identical to that for actin denaturation (*21*). Furthermore, we found that agarose-bound DNase I removed ANF from solution. In preparing for these experiments, we removed as much monomeric actin as possible from the ANF by rechromatographing the ANF-containing fractions. However, even after three cycles of column chromatography, monomeric actin was still the major band present on reduced SDS−PAGE, and the 140 kDa band was still the minor band. Using Western blot analysis, we did not detect α-actinin, C-protein, or gelsolin in our preparations. Of the antibodies reactive against skeletal muscle proteins that were available to us, the only antibodies reactive with the 140 kDa band were those against actin. Immunoreactivity with actin antibodies suggested that ANF might be an actin oligomer. However, from a number of experiments in which we monitored the kinetics of ANF-induced polymerization, it was apparent that ANF was not a preformed nucleus; in every case, a detectable lag occurred before the polymerization reaction reached its maximal rate. Thus, ANF is probably smaller than a trimer; however, the difference in the apparent molecular masses of ANF determined by gel filtration (84 kDa) and electrophoresis (140 kDa) was confounding. We used the ultracentrifugation technique of Bothwell et al. (*17*) (as described in Materials and Methods) to compare the sedimentation of ANF activity with the sedimentation of proteins with known molecular masses. The results of this experiment indicated that the molecular mass of ANF is in the 70−85 kDa range, in good agreement with the results from gel filtration. Thus, we concluded that ANF is most likely a covalently cross-linked actin dimer, and that its molecular mass of 140 kDa determined by SDS−PAGE is anomalous.

Millonig et al. (*5*) reported that two types of actin dimers were obtained from actin cross-linked with PBM, and these were denoted as upper and lower dimers according to their pattern of migration on SDS−PAGE. The upper dimer had an apparent molecular mass of 115 kDa as determined by reduced SDS−PAGE on 7.5% gels, while the lower dimer migrated with an apparent molecular mass of 84 kDa (*5*). A direct comparison between the electrophoretic mobilities of PBM-cross-linked actin and ANF on 10 and 7.5% reduced SDS−PAGE is shown in Figure 3. Clearly, both PBM-cross-linked upper dimers of actin and ANF have electrophoretic

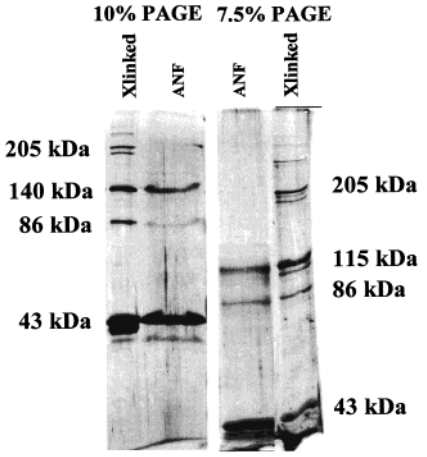

FIGURE 3: ANF and PBM-cross-linked actin migrate similarly on SDS−PAGE. Samples of PBM-cross-linked actin and ANF (from the ANF peak of a preparation chromatographed three times on Sephacryl S00 HR) were subjected to electrophoresis on both 7.5 and 10% SDS polyacrylamide gels. In each case, the molecular mass was determined by comparison to molecular mass standards.

mobilities that depend on the percentage of polyacrylamide in the gel. The simplest explanation for these results is that the frictional coefficients of upper dimers and ANF are equal and are greater than the frictional coefficient of lower dimers. Lower dimers, which are thought to have cysteine 374−cysteine 374 cross-links (*5*), are effectively linear chain molecules. However, upper dimers, known to be cysteine 374 cross-linked to lysine 191 (*22*), have a branched configuration. This branched configuration results in a higher frictional coefficient and slower electrophoretic mobility relative to those of the linear chain lower dimer.

Further support for the similarity between PBM dimers and ANF is shown in Figure 4. While monomeric actin elutes from DEAE chromatography columns at about 0.2 M KCl, ANF and PBM dimers both elute at higher salt concentrations. This may be attributable to the lower probability of simultaneous release from the substrate of both subunits of the dimer compared with the probability of release of a single monomer (i.e., more charge per molecule for dimers). Thus, ANF appears to be a cross-linked actin dimer, similar to the active upper dimer described by Millonig et al. (*5*).

ANF Formation. In the large number of preparations (>30) that we assayed for ANF activity, the ANF activity in solutions of monomeric actin appeared to be stable at 5 °C over a period of weeks. ANF activity in preparations that were cycled through three successive gel filtration columns appeared to be quantitatively recovered from each cycle. However, when solutions of F-actin prepared from fractions that contained no apparent ANF activity (trailing fractions from the gel filtration column) were incubated for long periods of time, an increase in the nucleation activity was noted. Figure 5 shows the increase with time in nucleation activity (plotted as ANF activity) for a sample of F-actin incubated at 22 °C. The ANF activity in samples with 0.5 mM DTT (△) suggests a minimal contribution from disulfide cross-linking. Figure 6 shows the level of formation of cross-linked actin dimers with increasing temperature. The reduced SDS−PAGE gel in Figure 6A shows that the intensity of the 140 kDa band characteristic of ANF, as well as a variety of other bands between 140 and 42 kDa, is increased as the sample is incubated at increasing temperatures. Figure 6B

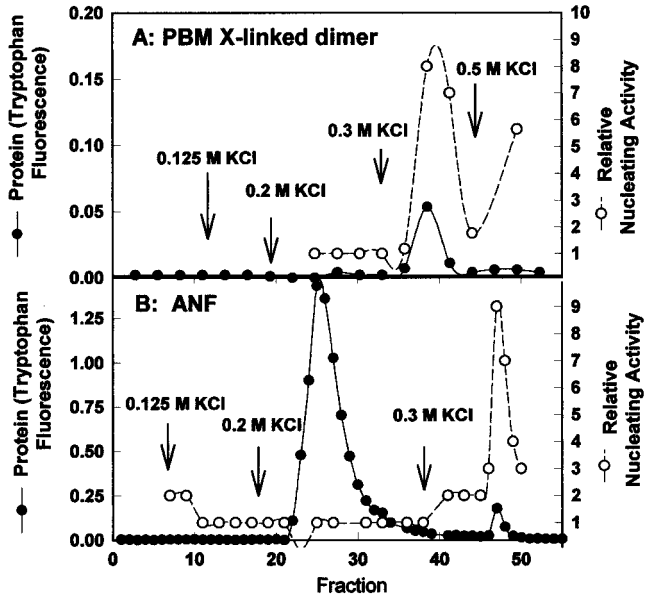

FIGURE 4: Separation of ANF or PBM-cross-linked dimers from monomeric actin using DEAE chromatography. Samples were applied to a 0.9 cm × 15 cm DEAE Sepharose FF column as described in Materials and Methods. After sample application and washing with buffer G, the column was eluted sequentially with buffer G containing 0.125 M KCl, 0.2 M KCl, and 0.3 M KCl. In the case of the PBM-cross-linked dimer, an additional step with 0.5 M KCl was used. Protein concentration (●, solid line) was monitored by intrinsic tryptophan fluorescence using actin as a standard. After removal of KCl by dialysis against buffer G, samples were assayed for actin nucleating activity (○, dashed line): (A) PBM-cross-linked actin containing primarily cross-linked dimers (some contamination by monomer and trimer was noted by SDS−PAGE analysis) and (B) a sample containing ANF.

shows that the relative ANF activity increases as a function of incubation temperature, in the same manner as does the intensity of the 140 kDa dimer band. The intensity of the 84 kDa band also increases, and it is not clear whether this species contributes to increased nucleation levels.

Origin of Actin Cross-Links. The formation of ANF in actin polymer solutions, but not monomer solutions, suggests that a modified amino acid residue within some of the monomeric subunits in the polymer can react with a neighboring subunit to form a covalently linked dimer. These modifications may arise in vivo, or during purification of the protein. To determine whether the actin was somehow modified during the actin purification procedures, we varied the methods used to prepare skeletal muscle acetone powder and purify actin. In one variation (described in the legend of Figure 7), we used a single acetone wash followed by lyophilization of the muscle residue for preparation of the acetone powder. Figure 7 shows that the Sephacryl S300 HR chromatography of actin treated with the usual five acetone washes (●) is similar to that purified from acetone powder prepared by a single acetone wash (▲). However, a broad peak of ANF activity (○) is present in actin prepared using the conventional five acetone washes, while the actin purified from acetone powder prepared with a single acetone wash had a markedly reduced ANF activity (△).

Reactive Carbonyls Introduced during Acetone Treatment of Muscle. The increase in ANF activity due to extended acetone washing (Figure 7) and the slow formation of ANF during prolonged incubation of F-actin shown in Figure 6 suggest that acetone treatment may introduce a reactive group

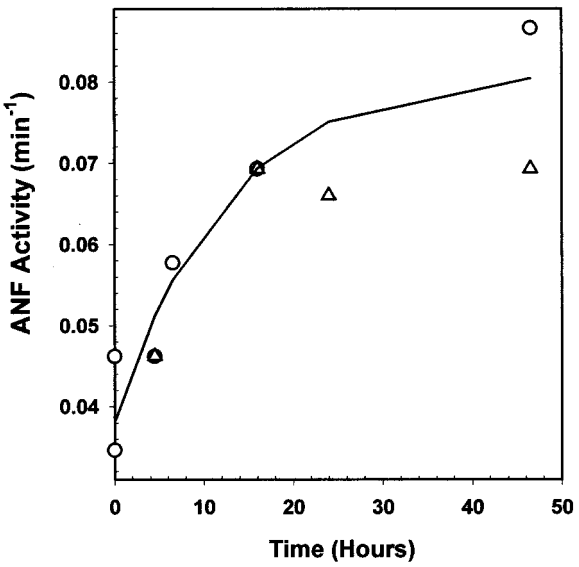

FIGURE 5: ANF forms spontaneously in solutions of F-actin. F-Actin (8.5 μM) from the trailing portion of a gel filtration column was incubated with (○) and without (△) 0.5 mM DTT at 22 °C. At various times, 1 mL aliquots were removed and placed in an ice bath. After the last sample had been removed (46.5 h), all of the samples were centrifuged at 280000*g* for 30 min, and the F-actin pellet was dissolved in G-buffer, diluted to a final actin concentration of 2 μM, and allowed to depolymerize overnight on ice. Polymerization was initiated and monitored as described in Materials and Methods except that in this experiment the actin concentration was 2 μM rather than 0.5 μM.

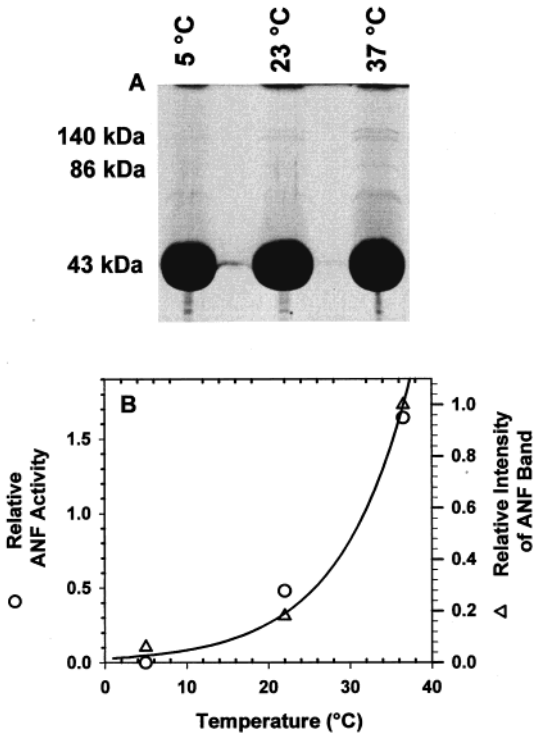

FIGURE 6: Temperature dependence of ANF formation. Samples of 12 μM F-actin prepared from column fractions lacking ANF activity were incubated at 5, 23, and 37 °C for 23.5 h. The samples were then brought to 5 °C and collected by centrifugation at 280000*g* for 30 min. The F-actin pellet was softened and homogenized with 25 μL of buffer G using a 200 μL pipet tip. An aliquot of 12.5 μL of this pellet was diluted with 12.5 μL of 2× Lammeli sample buffer and subjected to reduced SDS−PAGE. The remaining 12.5 μL was diluted to 1 mL with G-buffer and allowed to depolymerize for 20 h on ice before assaying for nucleating activity. (A) SDS−PAGE stained with Gel-Code Blue (Pierce, Rockford, IL). (B) Relative ANF activity (○) and staining intensity of the 140 kDa band (△).

into actin. This reactive group may then react with a neighboring subunit within the polymer to form a dimer. One of the possible modifications that we considered was the introduction of carbonyl moieties into actin. These modifications are relatively common and are known to occur in vivo through enzymatic deamination of lysine (*23*), through glycation when high concentrations of sugars are present (*24*), or through oxidation by a variety of reactive oxygen species. Therefore, we compared the carbonyl content of actin prepared from muscle tissue washed five times with acetone to that of actin purified from muscle tissue washed only once with acetone. (For once-washed tissue, the acetone was evaporated under a stream of nitrogen, and the remaining water was removed by lyophilization.) In addition, we determined the carbonyl content of β,γ-actin prepared from spleen tissue, which had not been treated with acetone. The carbonyl content of actin prepared by the standard procedure used in our lab results in 0.31 ± 0.09 carbonyl/actin molecule. The assay of multiple samples from the same preparation had a standard error of about 5%, while the variability between preparations was much larger (~30%), indicating that the carbonyl content varies with the preparation. In contrast, actin prepared from acetone powder washed only once with acetone contains only 0.07 ± 0.01 carbonyl/actin, a level similar to that found for β,γ-actin prepared from spleen without acetone treatment (0.05 ± 0.03 carbonyl/actin).

These results could indicate that, in our standard preparation, one in every three actin monomers contains a carbonyl moiety; alternatively, a smaller fraction of the actin could contain multiple carbonyl groups. Still another possible explanation is that a carbonyl-containing contaminant due to the acetone treatment could be copurified with the actin. If the presence of carbonyl groups were due to an impurity, the carbonyl-containing fraction would not be expected to migrate with the actin in the presence of SDS. We reacted actin with DNPH and subjected this actin to SDS−PAGE (shown in Figure 8, lanes 1 and 2). The DNP-modified carbonyls migrated with the actin, and actin prepared from acetone powder washed five times with acetone and containing 0.25 carbonyl/actin (lane 1) was much more reactive than that washed a single time with acetone and containing 0.07 carbonyl/actin (lane 2). Lanes 3 and 4 of Figure 8 show IEF of skeletal muscle actin containing 0.07 carbonyl/actin and actin containing 0.4 carbonyl/actin, respectively. It is apparent that the presence of carbonyl groups on the actin did not alter its isoelectric point. Lanes 5 and 6 of Figure 8 compare IEF of β,γ-actin from spleen containing 0.05 carbonyl/actin with skeletal muscle actin containing 0.4 carbonyl/actin. Note the expected higher p*I* of the non-muscle actin. Lanes 7 and 8 contain samples identical to those used in lanes 5 and 6 that were reacted with DNPH after IEF and transferred to PVDF membrane for Western blot analysis using antibody directed against DNP. We deduce from the IEF experiments that there is no subpopulation of carbonyl-containing actin with an altered isoelectric point.

The remaining possibilities are that a large percentage (~15−30%) of the actin molecules is modified with one or two carbonyl groups, or that there is a small percentage of

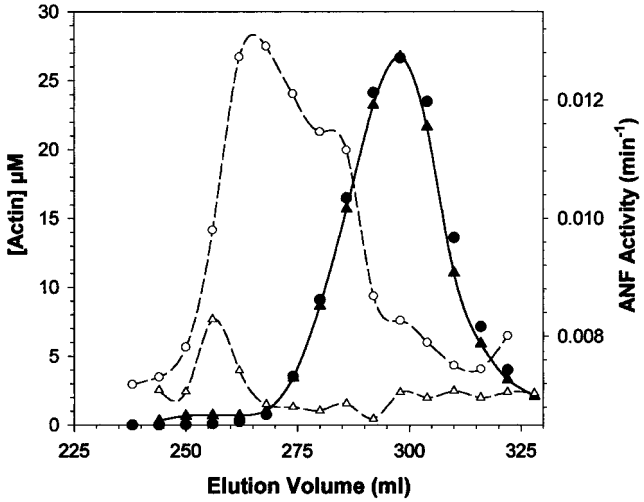

FIGURE 7: ANF activity and acetone washing during preparation of muscle acetone powder. Actin purified from traditionally prepared muscle acetone powder had gone through five acetone washes as described in the Materials and Methods (circles). The once-washed sample (triangles) was prepared by removing a portion of muscle residue after the first acetone wash and evaporating the acetone under a stream of nitrogen. The muscle residue was lyophilized to dryness and powdered in a Sorvall Omni-mixer. Matched concentrations of each actin preparation were gel filtered on a Sephacryl S300 HR column (as described in the legend of Figure 1) and assayed for protein concentration (black symbols, solid line) and ANF activity (white symbols, dashed line).

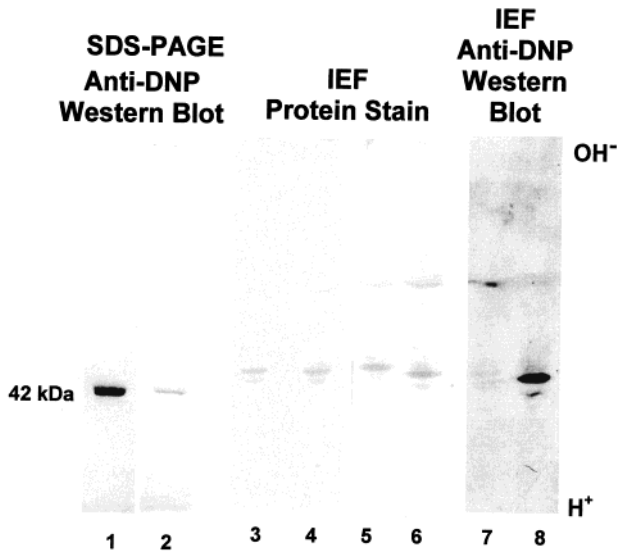

FIGURE 8: Detection of carbonyl groups in actin. In lanes 1 and 2, samples of 1 μg of skeletal muscle actin were reacted with DNPH, subjected to SDS−PAGE, transferred to PVDF membrane, and probed for DNP as described by Schacter et al. (*19*): lane 1, actin containing 0.25 carbonyl/actin; and lane 2, actin containing 0.07 carbonyl/actin. Lanes 3−6 show IEF of 4 μg of actin stained for protein using Sypro Ruby Red: lane 3, skeletal muscle actin containing 0.07 carbonyl/actin; lane 4, skeletal muscle actin containing 0.4 carbonyl/actin; lane 5, non-muscle β,γ-actin containing 0.05 carbonyl/actin; and lane 6, skeletal muscle actin containing 0.4 carbonyl/actin. Lanes 7 and 8 contain the same samples as lanes 5 and 6, respectively. The focused samples were reacted with DNPH, transferred to PVDF membrane, and analyzed for immunoreactivity with antibody against DNP.

the actin molecules containing multiple carbonyl groups. Figure 9 shows the mass spectra of actin containing either 0.07 or 0.4 carbonyl/actin. The mass spectrum for the sample containing 0.07 carbonyl/actin indicated a single protein with

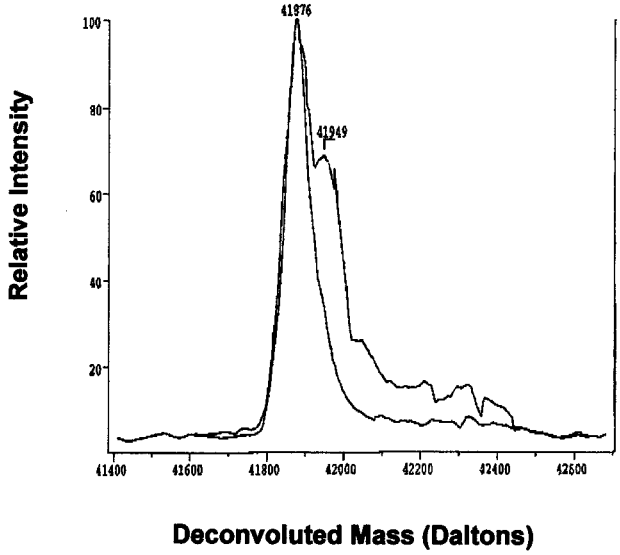

FIGURE 9: Mass spectrometry of actin with high and low carbonyl content. Deconvoluted electrospray ionization mass spectra were obtained as described in Materials and Methods. Actin containing 0.07 carbonyl/actin has a single peak at 41 896 Da, while actin containing 0.4 carbonyl/actin exhibits an additional shoulder with a mass of 41 949 Da. The expected average mass of skeletal muscle actin with N-terminal acetylation and methylation of histidine 73 is 41 872.9 Da.

a mass of 41 896 Da, while the mass spectrum for the sample containing 0.4 carbonyl/actin indicates that 30−40% of the protein has a mass 70−80 Da higher than that of actin. A mass spectrum (not shown) of actin containing 0.4 carbonyl/actin that had been reacted with DNPH exhibited a shift of the shoulder to a mass approximately 270 Da higher than that of actin, consistent with the binding of DNPH (molecular mass of 200 Da) to the carbonyl group on actin. From these data, it seems clear that most of the carbonyl-modified actin molecules contain a single carbonyl. Identification of the modification and the amino acid to which it is attached will require the higher resolution that can be obtained by MALDI-TOF mass spectrometry of proteolytic peptides of actin.

Correlation between the Carbonyl Content of Actin and the Level of ANF Formation. Actins containing high and low carbonyl content, prepared from acetone powder prepared with five and one acetone washes, respectively, were compared for their ability to form ANF on prolonged incubation as F-actin. Both samples were trailing edge fractions from the Sephacryl S300 HR chromatography column elutions of Figure 7 (elution volumes of 295−301 mL), and both contained low initial ANF activities. Actin from the muscle acetone powder washed once with acetone was determined to have 0.07 carbonyl/actin, and actin from the muscle acetone powder washed five times with acetone contained 0.26 carbonyl/actin. The samples were polymerized and incubated overnight at 5, 22, and 37 °C. Following complete depolymerization, these samples were assayed for ANF activity, and the results are shown in Figure 10. Actin containing 0.25 carbonyl/actin forms ANF in a temperature-dependent manner, in contrast to actin containing only 0.07 carbonyl/actin, which forms no significant amount of ANF. This experiment provides compelling evidence that the origin of the cross-link responsible for ANF activity is related to the presence of carbonyl groups introduced during the prolonged acetone treatment of skeletal muscle.

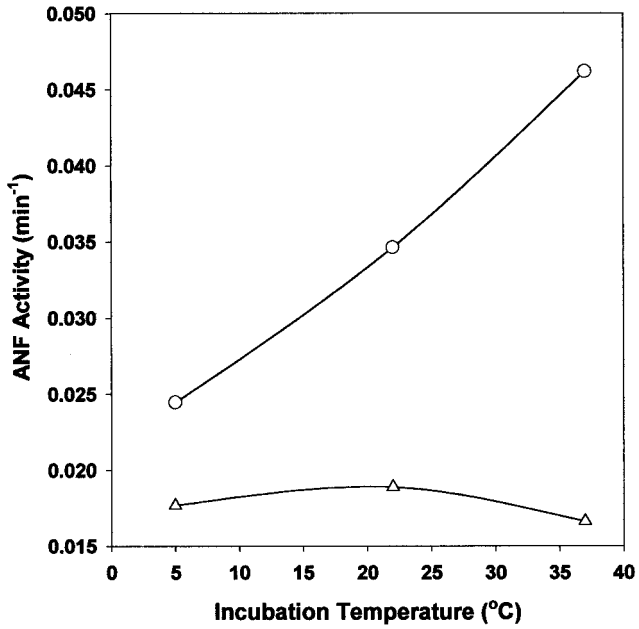

FIGURE 10: Correlation between actin carbonyl content and ANF formation. Actin prepared from muscle tissue washed five times with acetone and containing 0.25 carbonyl/actin (○) and actin prepared from muscle tissue washed once with acetone and containing 0.05 carbonyl/actin (△) were polymerized and incubated overnight at various temperatures. The F-actin was then collected by centrifugation at 280000*g* for 30 min, and the pellet was dissolved, diluted to a final concentration of 2 μM, and depolymerized by dialysis against buffer G for 16 h. The resulting G-actin was assayed for ANF activity.

DISCUSSION

Acetone Powder, Contaminants, and ANF. The procedure for preparing an acetone powder of muscle tissue dates back to the original work of Feuer and Straub (*25*). Most procedures in use today reference this work, although some modifications have been made. A. Szent-Gyorgyi modified this procedure slightly in 1951 (*26*), and in the early 1960s, A. G. Szent-Gyorgyi and A. Weber modified the method further for use in the Muscle Biochemistry course given at Woods Hole Marine Biological Laboratory (personal communication). To our knowledge, this procedure was never published, but is probably still widely used. A detailed description of this method (as used in our laboratory) is given in Materials and Methods. Carsten and Mommaerts published another modified procedure that eliminates pre-extraction of myosin (*27*), and this acetone powder is commercially available from Sigma Chemical Co. Many laboratories purify actin from this material. We did not exhaustively investigate the preparation of acetone powder, but in the few variations we did try, we found that pre-extraction of myosin and essentially complete lipid removal through multiple acetone washes resulted in the best actin yields. Actin prepared from acetone powder obtained from Sigma Chemical Co. gave poorer yields, and the amount of ANF was reduced. Reducing the number of acetone washes to a single wash was effective in reducing the number of carbonyl groups introduced into the actin, but the yield was only about 50% of that obtained from actin powder prepared with five acetone washes. In addition, lipid was present in the initial stages of purification of actin from muscle powder prepared with one acetone wash. On two occasions, we extracted actin from fresh muscle without acetone washes, which resulted in poor actin yields. In these preparations, leading edge samples from the Sephacryl S300 HR gel filtration peak that would be expected to contain ANF suppressed, rather than enhanced, actin polymerization rates. We attribute this suppression to the presence of CapZ and an apparent absence of ANF. Others (*2*−*4*) have reported the presence of CapZ in actin purified from muscle acetone powder and its difficult removal; Figure 2C shows that CapZ, a 76 kDa dimer, elutes in the same fraction as ANF, and complete separation by gel filtration is not possible.

Effects of ANF on Actin Polymerization Kinetics. The presence of cross-linked actin dimers in actin preparations can have dramatic effects on the polymerization characteristics of actin, as shown here, as well as on the length distributions of actin networks (*28*). Estimates of the dimer concentration in typical actin preparations varied, but in all cases were extremely low. The 0.5 μM actin solution used in the experiments depicted in Figure 1, for example, has a dimer concentration of only 0.14 nM (0.06% of the total actin by mass), as estimated from the polymerization half-time. Under slow nucleating conditions, even this small percentage of dimer has a dramatic impact on the polymerization kinetics. However, the low concentration relative to the total actin concentration makes characterization of the dimer difficult. While we have not exhaustively characterized the effects of ANF on actin polymerization kinetics, the results (not shown) of the experiments we have carried out are similar to results obtained using PBM-cross-linked actin dimers, which have been published by others (*10*, *11*). ANF appears to act as a nucleus precursor or stabilizer and is less effective when the nucleation rate is very slow [as is the case for low (<5 μM) CaATP−actin concentrations] because poor nucleation conditions generate too few nuclei for ANF stabilization. Most importantly, the k_{obs} for actin polymerization at fixed ANF concentrations is dependent on the actin monomer concentration. At actin concentrations with slow (limiting) nucleation rates, the presence of a preformed nucleus would be expected to result in polymerization rates that are independent of the actin concentration. Therefore, we conclude that ANF is not a preformed nucleus for actin polymerization.

ANF Is an Actin Dimer. Several lines of evidence lead to strong support for the identification of ANF as a cross-linked actin dimer. Direct comparisons between ANF and PBM-cross-linked dimers reveal similar polymerization kinetics, similar anomalous electrophoretic behavior, and similar elution profiles from DEAE chromatography media. The molecular mass of ANF is consistent with that of an actin dimer, and the reaction of ANF with antibodies specific for both the N- and C-terminal regions of actin supports the presence of actin in ANF. Nevertheless, we cannot rule out the possibility that an extraneous small protein or other moiety is present in ANF, perhaps in the cross-linked region.

ANF and Actin Carbonyls. We are not certain of the mechanism by which carbonyl groups are introduced into actin during acetone treatment of muscle. One possibility is that impurities in the acetone are responsible; the ACS-certified acetone used in our preparations, as well as all other high grades of acetone, is known to contain small amounts of aldehyde impurities (less than 0.002%). When the volumes of acetone used are considered, even small amounts of

impurities could be enough to result in protein modification. Levine et al. (*18*) noted an increase in the level of tritiated borohydride labeling of protein when acetone was used as a scavenger to destroy excess borohydride after protein reduction. These authors presumed that Schiff bases were formed between the protein and acetone. Our IEF experiment in Figure 8 suggests that the presence of carbonyl groups in actin did not alter its isoelectric point. For this reason, we believe that the introduction of the carbonyls into actin does not involve basic or acidic residues of actin. We do not yet have a good explanation and have not found a precedent for our observations in the literature.

As shown in Figure 10, when solutions of actin that previously contained low amounts of ANF but high carbonyl concentrations are polymerized and heated, ANF is formed. This is consistent with the idea that ANF is formed through reaction of carbonyl groups in the actin. However, until the actual cross-link is identified, we can only speculate as to the mechanism of its formation. The reaction appears to be very slow, and does not occur in solutions of monomeric actin at physiological temperatures. However, in related experiments in our laboratory (unpublished), we found that if a solution of monomeric actin is heated without reducing agents to 60−100 °C for a few minutes, covalent bonds form between actin monomers and more than 10 covalently cross-linked actin bands between 84 and 140 kDa are evident on reduced 10% SDS−PAGE gels. This large number of bands suggests that there is marked heterogeneity in cross-linking sites, with the formation of heterogeneous dimers having different frictional coefficients, probably due to the exposure of normally protected groups during thermal unfolding of the protein. The multiple cross-linked dimer bands observed in Figure 7B (especially at 37 °C) are most likely formed in a similar manner. These dimers do not appear to copurify with F-actin, and they are presumably inactive in enhancing actin nucleation. ANF activity seems to be associated with the "higher molecular weight" dimers, similar to the upper dimer described by Millonig el al. (*5*).

The cross-linking reaction responsible for dimer formation may be a reaction between carbonyl groups and the ϵ-amino group of lysine:

$$R_1CH{=}O + NH_2CH_2(CH_2)_2R_2 \rightarrow R_1CH{=}NCH_2(CH_2)_2R_2$$

or an aldol condensation between two carbonyl groups:

$$R_1CH{=}O + O{=}CH\text{-}CH_2\text{-}R_2 \rightarrow R_1\text{-}HC{=}C(HCO)\text{-}R_2$$

where R_1 and R_2 represent the two protein molecules. Cross-links of this type are known to occur in the structural proteins, collagen and elastin, where carbonyl groups are introduced by enzymatic post-translational modifications of lysine residues (*23*).

At physiological temperatures, actin cross-links appear to form only in polymer solutions; it may be that the steric arrangement of subunits within the polymer structure facilitates their formation. Because the cross-linked dimer is typically present at sub-nanomolar concentrations and is always in the presence of a relatively large concentration of monomeric actin, we have not yet been successful at determining the isoelectric point of ANF. If the cross-link involves lysine residues, we might expect a shift to a lower isoelectric point.

An important product of the carbonyl-induced cross-linking reaction is ANF, an active actin dimer that affects the polymerization rate of actin. However, merely the presence of carbonyl groups on actin monomers may have additional effects on actin chemistry. Alteration of the interaction between actin and other proteins, changes in the physical properties of actin filaments, and direct effects on cross-linking of actin filament networks are possible. Until homogeneous preparations of modified and unmodified actins are obtained, it may be difficult to clearly determine the effects of carbonyl formation on the properties of actin. While cross-linked dimers may be removed from the preparation by extensive gel filtration methods, we have thus far been unable to separate carbonyl-containing actin from unmodified actin. In the absence of a good technique for such a separation, it is worth considering methods that reduce the extent of introduction of carbonyl groups into actin. As has already been discussed, reducing the number of acetone washes during preparation of muscle acetone powder yields actin with very low carbonyl content. Although the procedure reduces the yield of actin substantially, the yield of actin is still quite good. Recently, we noted that including 1 mM PMSF, 20 μg/mL aprotinin, and 20 μg/mL cystatin during the extraction improved the yield of actin, and approximately 120 mg of actin was obtained from 6.6 g of the acetone powder washed once. Complete elimination of acetone treatment is possible; however, the yields of actin are lower, and the convenience of storage as a dry powder is sacrificed. Alternate sources and preparations of actin may also be considered. Recently, our laboratory has used β,γ-actin derived from spleen, and we have included the purification methodology for preparing milligram quantities of spleen actin in Materials and Methods.[3] Other methods for preparing actin from muscle tissue may be developed, and it may be possible to dehydrate and remove lipid from muscle using other solvents, such as ethanol or ether. Alternatively, Kuroda (*29*) reported a method that eliminates acetone treatment whereby the actin is extracted from myofibrils after removal of myosin.

Physiological Effects. The physiological significance of the findings presented here is not clear. Any modified actin with abnormal functional characteristics could have a significant impact on the integrity of the actin-based cytoskeleton. Such an impact could arise from a direct effect on the structural integrity of actin filaments, or from an alteration in the interactions between actin and other proteins. The existence of oxidized actin within normal endothelial cells and an increase in the level of oxidized actin upon treatment with TNF-α have been reported (*6*). Moreover, Dalle Donne et al. (*30*) have reported alterations in the polymerization characteristics of actin oxidized by H_2O_2.

In vivo, carbonyl formation is believed to occur in proteins near a divalent cation-binding site (*31*). A cation capable of redox cycling is required, typically iron or copper, and the

[3] The initial cost of preparing the polyproline Sepharose is high (~$1500); however, the medium is reusable indefinitely if properly cared for, and the procedure does not require the expense associated with acquisition and sacrifice of rabbits.

oxidation occurs at specific amino acids in proximity to the metal binding site (*31*). Actin is known to have several divalent cation-binding sites, and while Mg^{2+} is thought to be the cation bound to actin in vivo, it is conceivable that iron or copper could compete for these sites. Another in vivo modification worth consideration is glycation of lysine residues at high concentrations of reducing sugars, such as by glucose in diabetes (*23*). Such modified actin molecules could be precursors for dimer formation.

The work reported here has alerted us to the presence of cross-linked actin dimers that can affect studies of actin kinetics. However, we are now also aware of the possibility that in an in vivo environment containing a complex mixture of carbohydrates, reactive oxygen species, and oxidase enzymes, reactions leading to dimer formation may well occur. Further work will be necessary to fully determine whether oxidation of actin in vivo is significant and whether such oxidation leads to formation of actin dimers within the cell.

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