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In Vitro Labeling of Proteins by Reductive Methylation: Application to Proteins Involved in Supramolecular Structures

C.S. Heacock, B.W. Bernstein, A.S. Duhaiman, D.A. Amorese, and J.R. Bamburg

Department of Biochemistry and Graduate Program of Cellular and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523

Actin and tropomyosin, purified from both muscle and brain, and α-actinin, purified from muscle, have been labeled in vitro by reductive methylation to specific activities of greater than 10^5 dpm/μg protein. Actin so modified bound DNase I and polymerized identically to unmodified actin. Furthermore, the spectral properties of actin did not change after labeling. The interactions of labeled tropomyosin and α-actinin with F-actin were nearly identical to those of the unmodified proteins. These modified proteins comigrated with their unmodified counterparts in both SDS-containing polyacrylamide gels and isoelectric focusing gels. The labeled actin was quantitatively extracted from SDS-containing polyacrylamide gels (yield > 98% of radioactivity applied demonstrating that all of the radioactivity was protein bound. The reductive methylation procedure worked well at pH 8.0–8.5 in either pyrophosphate buffer or Bicine buffer using formaldehyde with [^{3}H]-sodium borohydride as the reducing agent. The procedure could also be performed at pH 7.0 in phosphate buffer using [^{14}C]-formaldehyde with sodium cyanoborohydride as the reducing agent. Proteins so labeled are ideal for use in quantitative experiments involving protein-protein interactions.

Key words: actin, tropomyosin, α-actinin, reductive methylation, microfilament assembly, DNase I inhibition

Quantitative measurements of the interaction between cytoskeletal proteins have employed many probes including fluorescent [1–3] and spin labels [4,5]. However, for many biochemical methods of analysis radiolabeled proteins are more useful. While many specific procedures have been developed for labeling a functional group of an isolated cytoskeletal protein without loss of biological activity [6–9], the most general procedure used for labeling these proteins to a high specific activity is the incorporation of ^{125}I via the lactoperoxidase method [10,11]. The drawback of this method is that a product with a relatively short halflife is obtained. In vivo labeling of proteins using [^{35}S]-labeled amino acids can give a product with reasonably high specific activity [12,13] if the protein has a relatively rapid turnover.

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However, in vivo labeling is generally an inefficient and costly procedure and is not applicable to many proteins with long turnover times.

To avoid these problems and to obtain native cytoskeletal proteins of high specific activity, we used a modification of the reductive methylation procedure first described by Means and Feeney [14] and further studied by Rice and Means [15], Nelles and Bamburg [16], and Geoghegan et al [17]. This method has the advantage of allowing the incorporation of either a ^{3}H or a ^{14}C label using [^{3}H]-sodium borohydride or [^{14}C]-formaldehyde. Recently a similar procedure was used to obtain tritium-labeled proteins for radioimmunoassay [18].

The native character of a modified enzyme is typically checked by measurement of its specific enzymatic activity. This test is sensitive to the conformation of the protein but not necessarily to surface charge modifications which could affect protein–protein interactions. Even antibody binding to modified proteins tests only small, specific regions of the surface for native conformation. A labeling procedure that leaves an enzyme with close to normal activity could denature a structural protein. The binding interactions of proteins involved in supramolecular structures are a more rigorous test for perturbations in overall structure and function because these interactions involve large regions of the surface of the protein and, therefore, probably are more sensitive to the slight changes induced by modification.

In this study the cytoskeletal proteins, actin, tropomyosin, and α-actinin, are labeled with ^{3}H or ^{14}C by reductive methylation. These modified proteins are then subjected to an analysis of their ability to interact with other cytoskeletal proteins.

METHODS

Materials

[^{3}H]-Sodium borohydride (16–22 Ci/mmole) was purchased from Research Products International Corp. (Mt. Prospect, Illinois) in 25 mCi amounts. The entire vial was dissolved in 10 mM NaOH, divided into aliquots, lyophilized in small vials, and stored at −20°C. [^{14}C]-Formaldehyde (50 mCi/mmole), obtained from New England Nuclear (Boston, Massachusetts) in 250 μCi amounts, was diluted to 4 mM in 12% methanol and frozen in small aliquots at −20°C. DNase I and Bicine were obtained from Sigma (St. Louis, Missouri). All other chemicals were reagent grade.

Protein Purification

Rabbit skeletal muscle actin was purified according to Spudich and Watt [19] with an additional depolymerization-polymerization cycle. Chick embryo brain actin was isolated according to Pardee and Bamburg [20] with an additional cycle of polymerization.

Rabbit muscle tropomyosin was prepared according to Greaser and Gergely [21] with the addition of an hydroxyapatite column as a final step [22]. Muscle tropomyosin was stored at 4°C in 5 mM Tris (pH 7.5), 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol, 2 mM NaN_3 (TM storage buffer). Bovine brain tropomyosin was prepared from brain acetone powder using modifications of the methods of Fine et al. [23] and der Terrossian et al. [24]. After extraction of the powder with 10 mM imidazole (pH 7.0), 1 M KCl, 1 mM dithiothreitol, and 2 mM NaN_3 (20 ml/gm powder) for 24 hr at room temperature, the particulate material was removed by centrifugation at 10^5 *g* for 2 hr. All subsequent steps were carried out at 4°C. The super-

natant solution was titrated to pH 4.1 with acetic acid and centrifuged 1 hr at 4×10^4 *g*. Tropomyosin was extracted from the pellet by resuspending and stirring the pellet for 4–12 hr in the extraction buffer titrated to pH 8.0. After removal of denatured protein by centrifugation at 4×10^4 *g* for 1 hr, the tropomyosin was precipitated again by addition of 10 volumes of 95% ethanol. The precipitate was collected by centrifugation and resuspended in extraction buffer by stirring overnight. The solution was clarified by centrifugation and taken to 40% saturation with ammonium sulfate, the precipitate removed by centrifugation at 4×10^4 *g* and the supernatant dialyzed against extraction buffer. The sample was applied to an hydroxyapatite column (1.5 cm × 5 cm) equilibrated in 1 mM potassium phosphate (pH 7.0), 1 M KCl, 0.5 mM dithiothreitol, and 2 mM NaN_3 [24]. The tropomyosin was eluted with a linear gradient of the same buffer containing 1–200 mM potassium phosphate. Fractions containing tropomyosin were identified by electrophoresis on SDS-containing polyacrylamide gels. The pooled tropomyosin-containing fractions were concentrated with Aquacide II-A (Calbiochem-Behring Corp., La-Jolla, California), made 10 mM in EDTA, and then dialyzed against 10 mM Tris (pH 7.5), 5 mM KCl, 0.5 mM dithiothreitol, and 2 mM NaN_3. The brain tropomyosin was stored at 4°C.

Chicken breast muscle α-actinin was purified by a modification of the method of Feramisco and Burridge [25]. Muscle (100 gm) was minced, washed in 10 volumes of ice cold 0.5 mM phenylmethylsulfonyl fluoride (PMSF) solution, and centrifuged at low speed. α-Actinin was extracted from the pellet by homogenization in 10 volumes of 2 mM Tris-acetate (pH 9.0), 1 mM EGTA, 0.5 mM PMSF, and 2 mM NaN_3 for 40 min at room temperature. After centrifugation, the α-actinin was precipitated with ammonium sulfate (10–40% fraction). The ammonium sulfate precipitate was collected by centrifugation, resuspended in and dialyzed against 50 mM potassium phosphate (pH 7.1) and applied to an hydroxyapatite column (5 cm × 2.5 cm) equilibrated in the same buffer. The α-actinin was eluted with a linear gradient of 50–500 mM potassium phosphate (pH 7.1). Fractions containing α-actinin were identified by electrophoresis on SDS-containing polyacrylamide gels, pooled, and concentrated by ammonium sulfate precipitation (0–43%). The precipitate was dissolved in 20 mM Tris-Cl (pH 7.6), 0.2 mM dithiothreitol, 2 mM NaN_3, and applied to a column of Sepharose CL-6B (2.0 × 95 cm) equilibrated in the same buffer. The α-actinin-containing fractions were identified as above and concentrated by ultrafiltration on an Amicon PM-30 membrane. The α-actinin was stored at 4°C.

Labeling Procedure

Prior to labeling, actin and tropomyosin were dialyzed against 0.1 M sodium pyrophosphate (pH 8.5). α-Actinin was denatured by pyrophosphate buffer; therefore 0.1 M Bicine (pH 8.0) was used instead. Sufficient formaldehyde was added to the protein to achieve a given ratio of formaldehyde to total amino groups (see Fig. 2), and adequate sodium borohydride was added to reduce all of the formaldehyde (slightly exceeding one-quarter the amount of formaldehyde). These reagents were added to capped vials in a well ventilated hood at room temperature in fractional doses with a short incubation period between additions. For example, to 2.8 mg F-actin in 1 ml (6.6×10^{-5} M), was added 50 μl 13 mM formaldehyde; after a 5 min incubation, 20 μl 2 mM $NaBH_4$ (22 Ci/mmole) was added, and this addition was repeated four times with 3 min intervals between additions. The cycle of addition of

formaldehyde and $NaBH_4$ was repeated again and the sample was incubated 5 min. This procedure resulted in final concentrations of 0.05 mM actin (1 mM amino groups), 1.0 mM formaldehyde, 0.3 mM [^{3}H]-$NaBH_4$ (22 Ci/mmole) and a final specific activity of the actin of about 10^5 dpm/μg with about 2% of the total amino groups labeled. Similar reagent quantities were used for 2.4 mg α-actinin and 0.7 mg tropomyosin (final concentration of 1 mM amino groups). For [^{14}C]-labeling, the above procedure was employed with the inclusion of [^{14}C]-formaldehyde. However, with the substitution of sodium cyanoborohydride for the $NaBH_4$, the labeling reaction could be performed with equal efficiency at pH 7.0 in 0.1 M sodium phosphate.

After labeling, all proteins were either dialyzed or centrifuged and resuspended in suitable buffers in order to remove all unreacted reagents. Actin, after labeling as F-actin, was centrifuged 10 min at 170,000 *g* at 4°C in a Beckman Airfuge. The pellet was resuspended in 0.1 M sodium pyrophosphate (pH 8.5), recentrifuged, and resuspended in and dialyzed against 2 mM Tris-Cl (pH 8.0), 0.2 mM Na_2-ATP, 0.5 mM dithiothreitol, 0.2 mM $CaCl_2$ at 4°C overnight to depolymerize the actin to its monomeric state. The sample was centrifuged as above and the actin in the supernatant assembled by addition of concentrated stock salt solutions to 4 mM $MgCl_2$, 0.1 M KCl. After 2 hr at room temperature, the viscous sample was centrifuged, and the pellet resuspended and dialyzed overnight as above. The sample was then centrifuged, and the supernatant was used as G-actin, or the G-actin was reassembled and pelleted as above and used as F-actin.

Labeled tropomyosin was removed from reagents by precipitation in 10 volumes of 95% ethanol at 4°C followed by centrifugation at 1000 *g* and resuspension of the tropomyosin pellet in and dialysis against the storage buffer.

Labeled α-actinin was separated from labeling reagents by extensive dialysis against 20 mM Tris (pH 7.6), 1 mM NaN_3, 0.2 mM dithiothreitol.

Protein Determination

G-Actin from both rabbit skeletal muscle and chick embryo brain was quantitated using both $E^{1\%}_{290\,nm,\,1\,cm} = 6.3$ [26,20] and a DNase I binding assay [27, 28]. Concentrations of muscle tropomyosin and α-actinin in stock solutions were determined spectrophotometrically using $E^{1\%}_{278nm,\,1\,cm} = 2.9$ [29] and $E^{1\%}_{280nm,\,1\,cm} = 9.7$ [30], respectively. The Bio-Rad microassay (Bio-Rad Laboratories, Richmond, California) was used to quantitate brain tropomyosin, employing muscle tropomyosin as a standard.

Actin Assembly and Electron Microscopy

G-Actin was chromatographed at 4°C on a column of Sephadex G-50 (1 × 30 cm) to equilibrate the actin with the 2 mM Tris-Cl, 0.1 mM ATP, 0.2 mM dithiothreitol buffer at pH 7.6. Assembly of the actin peak was monitored by measuring the increase in absorbance at 232 nm [31] following the addition of salts to a final concentration of 4 mM $MgCl_2$, 0.1 M KCl. After about 1 hr at room temperature, samples were removed from the cuvettes, diluted to about 0.1 mg/ml, placed on Formvar-coated 400-mesh grids, stained with filtered 1% uranyl acetate, and viewed with a Philips 200 electron microscope.

Tropomyosin and α-Actinin Binding to F-Actin

Muscle [^{3}H]-tropomyosin binding to muscle F-actin was determined by incubating a mixture of [^{3}H]-tropomyosin and tropomyosin with F-actin at room temperature for 16 hr. Different specific activity mixtures and different weight ratios of tropomyosin/F-actin were used. The [^{3}H]-tropomyosin binding to F-actin was determined by comparing the difference in radioactivity in the solution before centrifuging with the radioactivity in the supernatant after centrifuging (170,000 *g* for 10 min in a Beckman Airfuge). The amount of unlabeled tropomyosin bound to F-actin was determined by densitometry of SDS-containing polyacrylamide gels of the solution before and after centrifugation. A tropomyosin standard curve was prepared, and internal standards of tropomyosin were included on each gel in order to normalize variations in staining intensity. Without addition of F-actin less than 2% of the tropomyosin sedimented.

The binding characteristics of α-actinin to F-actin were determined by incubating a constant amount of F-actin with increasing concentrations of [^{3}H]- α-actinin. After centrifugation to pellet the F-actin and bound α-actinin, the pellets were resuspended in water and aliquots were removed for liquid scintillation counting. Aliquots of both supernatant and pellet fractions were also prepared for SDS-containing polyacrylamide gel electrophoresis in order to determine the amount of F-actin which was recovered in each pellet. Internal standards of both α-actinin and actin were applied to each SDS gel in order to normalize for variations in staining. Peak areas were determined by densitometry. For measuring binding of unlabeled α-actinin to F-actin the same procedure was followed except that all quantitation was done by densitometry of samples subjected to electrophoresis and staining in SDS gels and comparison to an α-actinin standard curve. Corrections were made for the small amount ($< 2\%$) of the α-actinin that sedimented under identical conditions without actin present.

Liquid Scintillation Counting

Radioactivity was quantitated in a Packard Tricarb Scintillation counter. Aqueous samples were solubilized in Protosol (New England Nuclear) before a toluene-based cocktail containing 0.01% 1,4-*bis* [2-(5-phenyloxazolyl)] benzene and 0.5% 2,5-diphenyloxazole (Fisher Scientific Company) was added. Counting efficiencies, as determined by internal standards, were 20–25% for ^{3}H and 80% for ^{14}C.

Gel Electrophoresis and Fluorography

Homogeneity of the proteins was determined by SDS-PAGE on 7.5, 10, or 12.5% acrylamide slab gels using 0.1 M Tris-Bicine buffer (pH 8.3) containing 0.1% SDS. Electrophoresis of radioactive samples (about 10 ng) was performed on 0.1% SDS-containing, discontinuous 12% acrylamide gels [32]. Gels were stained with Coomassie blue, destained, swollen, and photographed before preparation for fluorography. For densitometry studies, gels were scanned at 540 nm with an ISCO slab gel scanner. Isoelectric focusing slab gels were prepared according to O'Farrell [33]. A section of the focused gel was cut into 3-mm slices, soaked in water, and the pH gradient determined. The rest of the gel was soaked in 10% trichloroacetic acid, washed in 50% methanol–10% acetic acid, stained with Coomassie blue, destained, and photographed.

Fluorographs were obtained by soaking the gels in 10 volumes 100% dimethylsulfoxide (DMSO) for 30 min with one change, then 5 volumes of 20% 2,5-diphenyloxazole in DMSO at room temperature for 3 hr, followed by water to swell the gel to original size [34]. Gels were dried over a heated vacuum gel dryer, and directly exposed to Kodak XR-5 film at −70°C for 2–96 hr.

Recovery of Radioactive Protein From Gel

Labeled actin was mixed with different amounts of unlabeled actin and triplicate samples were electrophoresed on an SDS-containing 8.5% acrylamide gel (Tris-Bicine system). The gel was stained with 0.5% Page blue G 90 (BDH Biochemicals) in 50% methanol, 10% acetic acid, destained in the same solution without dye, and the stained bands removed and soaked in 100 μl 1% SDS for 1 hr at room temperature. The swollen gel slices were homogenized and the homogenate centrifuged in a Beckman Microfuge for 2 min. The supernatant was removed and the pellet homogenized in an additional 100 μl 1% SDS, incubated for 10 min, and centrifuged. This step was repeated twice more, giving 400 μl of each extract. Fifty μl of each supernatant (in triplicate) and pellet (in duplicate) were solubilized overnight at 25°C in 300 μl Protosol, and radioactivity was measured by liquid scintillation counting with internal standards used to correct for quenching.

High Performance Liquid Chromatography

HPLC was performed on a 0.7 × 30 cm gel filtration column (Protein I-125, Waters Assoc.) equilibrated in 10 mM Tris, 0.1 M NaCl (pH 7.4), operating with a flow rate of 1 ml/min. Effluent was monitored at 210 nm with an ISCO absorbance monitor model 1840 operated at 1 cm/min, 0.2 absorbance full scale. The column was calibrated with bovine serum albumin (monomers and higher oligomers), ovalbumin, actin, DNase I, myoglobin, Na_2-ATP, and NaN_3. Standards were run before and after each sample. The variation in elution times was less than ± 0.1 min. Samples were mixed to a final volume of 70 μl of which 45 μl was injected into a 20-μl sample loop before loading. Fractions were collected for determination of radioactivity by liquid scintillation counting.

RESULTS

To insure the ability of actin to self-assemble, actin was routinely labeled in its filamentous form (F-actin) and cycled through disassembly and reassembly before further use. The yield of labeled actin was about 80% after cycling, which is similar to yields for unmodified actin with optimal results obtained using about 1 mg/ml throughout.

In order to show that the labeled protein is not denatured selectively and that it retains its native characteristics, it is necessary to demonstrate that the specific activity of the purified protein does not differ from the material lost during the postlabel recycling. Therefore, following the first dialysis step after labeling in which no protein had yet been discarded, actin was separated by HPLC gel filtration into its characteristic monomeric and oligomeric fractions (Fig. 1a), both of which had similar specific activities. When excess DNase was incubated with the sample for several minutes before chromatography, the actin oligomer peak disappeared (Fig. 1b) and was replaced by a single peak of radioactivity corresponding to

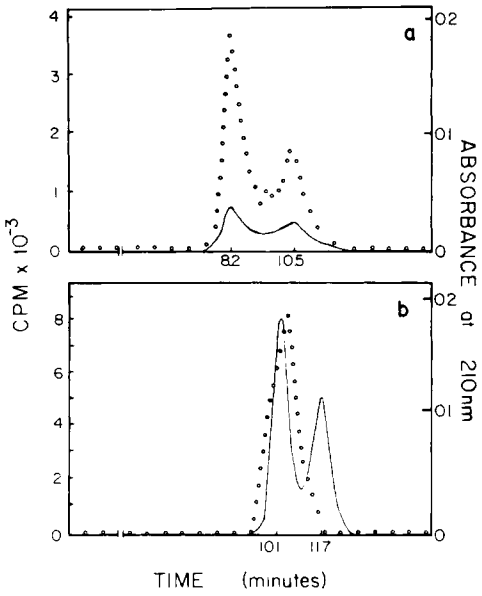

Fig. 1. HPLC profile of labeled rabbit muscle actin. (a) [^{3}H]-actin (1.2×10^5 dpm/μg) after first depolymerization subsequent to labeling (see Methods) plus DNase buffer. (b) [^{3}H]-actin plus DNase (about four-fold excess) after 10 min at room temperature. Note absence of actin oligomer (void volume) peak. Elution time in minutes from injection: void volume (8.2 min); DNase-actin complex (10.1 min); actin monomer (10.5 min); DNase (11.7 min); NaN_3 (14.2 min). $A_{210\ nm}$ (———); cpm (○) with counting efficiency of 24.7%.

a DNase-actin monomer complex. The actin in this peak had the same specific activity as both the monomer and oligomer fractions analyzed in Figure 1a indicating that all of the actin oligomers are competent to bind and be depolymerized by DNase [35]. Therefore, the actin lost during postlabel recycling does not appear to be protein that was denatured selectively by the labeling procedure.

When a fixed ratio of [^{3}H]-sodium borohydride to actin was used in the labeling procedure, increasing the amount of formaldehyde gave increasing specific activities of the labeled actin (Fig. 2). The specific activities of actin cycled after labeling were determined using the DNase I binding assay to quantitate total actin. The validity of using the DNase I assay for the labeled actin was verified by the identity of standard curves obtained for the modified and unmodified protein (Fig. 3), which also demonstrated that the labeled actin retains its ability to inhibit DNase I. Labeled G-actin is able to self-assemble into microfilaments with similar kinetics and to similar extents as unlabeled G-actin, as measured by an increase in absorbance at 232 nm (Fig. 4). The morphology of the filaments obtained with modified or unlabeled actin is identical (Fig. 5).

Figure 6 shows the muscle tropomyosin saturation curve of muscle F-actin. The binding characteristics of [^{3}H]-tropomyosin and unlabeled tropomyosin taken from the same mixtures are nearly identical; both species saturate F-actin at approximately 80% of the theoretical stoichiometry and require approximately a 0.23 molar ratio of tropomyosin to F-actin to achieve saturation. Both species also show

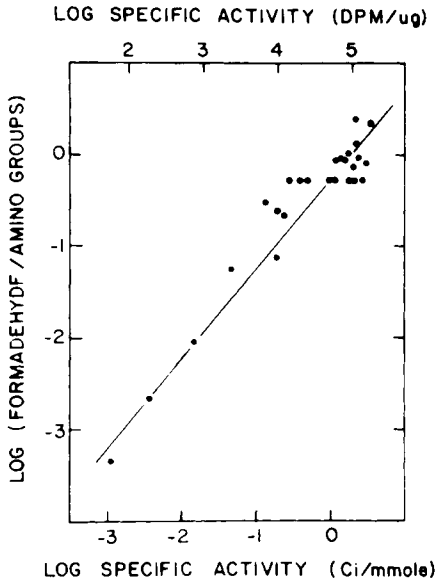

Fig. 2. Effect of variation in the formaldehyde/amine ratio of the labeling reaction on the specific activity of actin. The formaldehyde and amine concentrations are plotted using the concentrations at the end of the labeling procedure. All the data are for rabbit muscle actin using 20 amines per molecule. Each point represents one labeling experiment. Similar data were obtained utilizing actin from chick embryo brain.

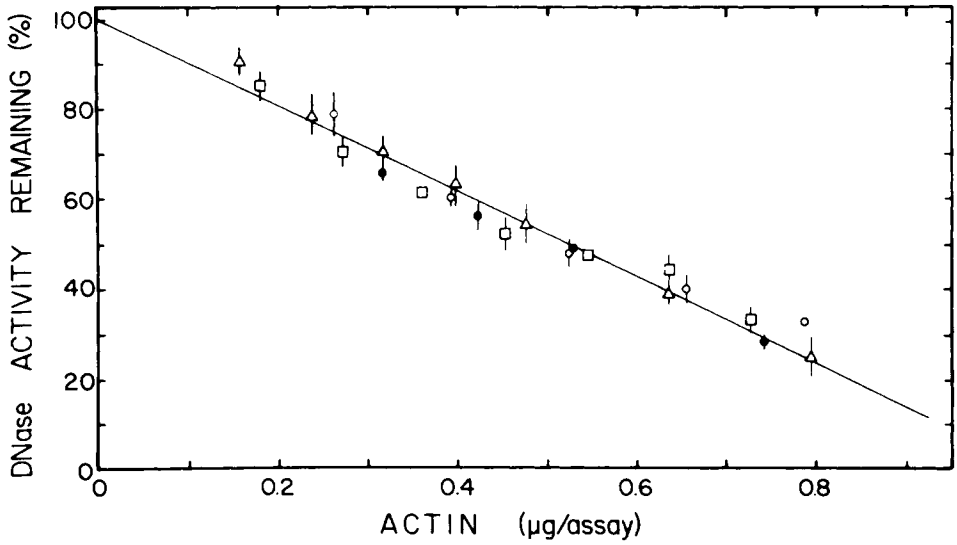

Fig. 3. Inhibition of DNase by [^{3}H], [^{14}C], and control rabbit muscle actin. G-Actin concentrations were determined after passing each sample through a 0.5 × 30 cm Sephadex G-50 column to equilibrate the sample with dialysis buffer. (△) [^{3}H]-actin, 1.1 × 10^5 dpm/µg; (□) [^{14}C]-actin, 21 dpm/µg; (●) unmodified actin cycled with samples; (○) unlabeled actin freshly purified. Each sample and buffer control was assayed in quadruplicate; the error bars show one standard deviation. Similar data were obtained utilizing actin from chick embryo brain.

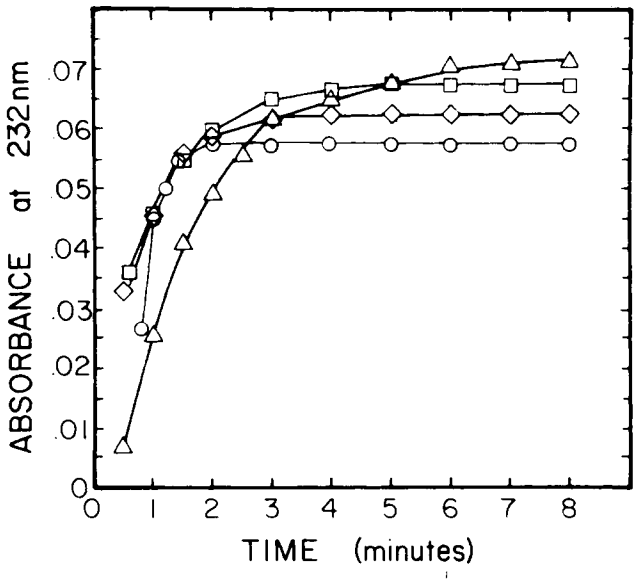

Fig. 4. Self-assembly of [^{3}H], [^{14}C], and control rabbit muscle actin. Values were normalized to 0.3 mg/ml with actual concentrations ranging from 0.28–0.36 mg/ml. (○) [^{3}H]-actin, 1.1 × 10^5 dpm/μg; (△) [^{14}C]-actin, 32 dpm/μg; (◇) modified nonradioactive actin; (□) unmodified actin cycled with samples.

the typical cooperative binding characteristics (Fig. 6, inset) reported for unlabeled tropomyosin [36, 37]. The selection of binding conditions was based on the report of Yang et al [37]. F-Actin incubated with saturating levels of tropomyosin was observed by electron microscopy to have the apparent reduced flexibility previously reported [38].

Figure 7 shows the saturation curve of F-actin with both unlabeled and [^{3}H]-α-actinin. The binding characteristics of the modified protein appear to be nearly identical to the control with both species achieving saturation at a molar ratio of approximately 1 α-actinin to 10 actin monomers.

The electrophoretic mobility of the modified proteins in SDS-polyacrylamide gels does not differ from that of the unlabeled proteins (Fig. 8A) indicating no detectable change in molecular weight. Recovery of label from actin bands was greater than 98% of the protein loaded (1.1, 5.9, and 14 μg samples), demonstrating that all of the label was covalently attached to the protein.

Demonstration of the identity of isoelectric points of proteins before and after labeling indicates that the added methyl groups do not detectably alter the overall charge of the proteins (Fig. 8B). However, a protein whose isoelectric point is closer to the pK_a of the lysine ϵ-amino group ($\sim$10.5) might show a small change in its isoelectric point upon methylation since the pK_a of the ϵ-N,N-dimethyllysine is about 0.5 pH unit lower than its nonmethylated counterpart [14].

Labeling of a protein preparation can also give some information as to its homogeneity. By lengthening the time of fluorography of the SDS gels containing the labeled protein, it is possible to detect low levels of impurities that do not show up by conventional staining procedures. Fluorographs of the same gel of brain

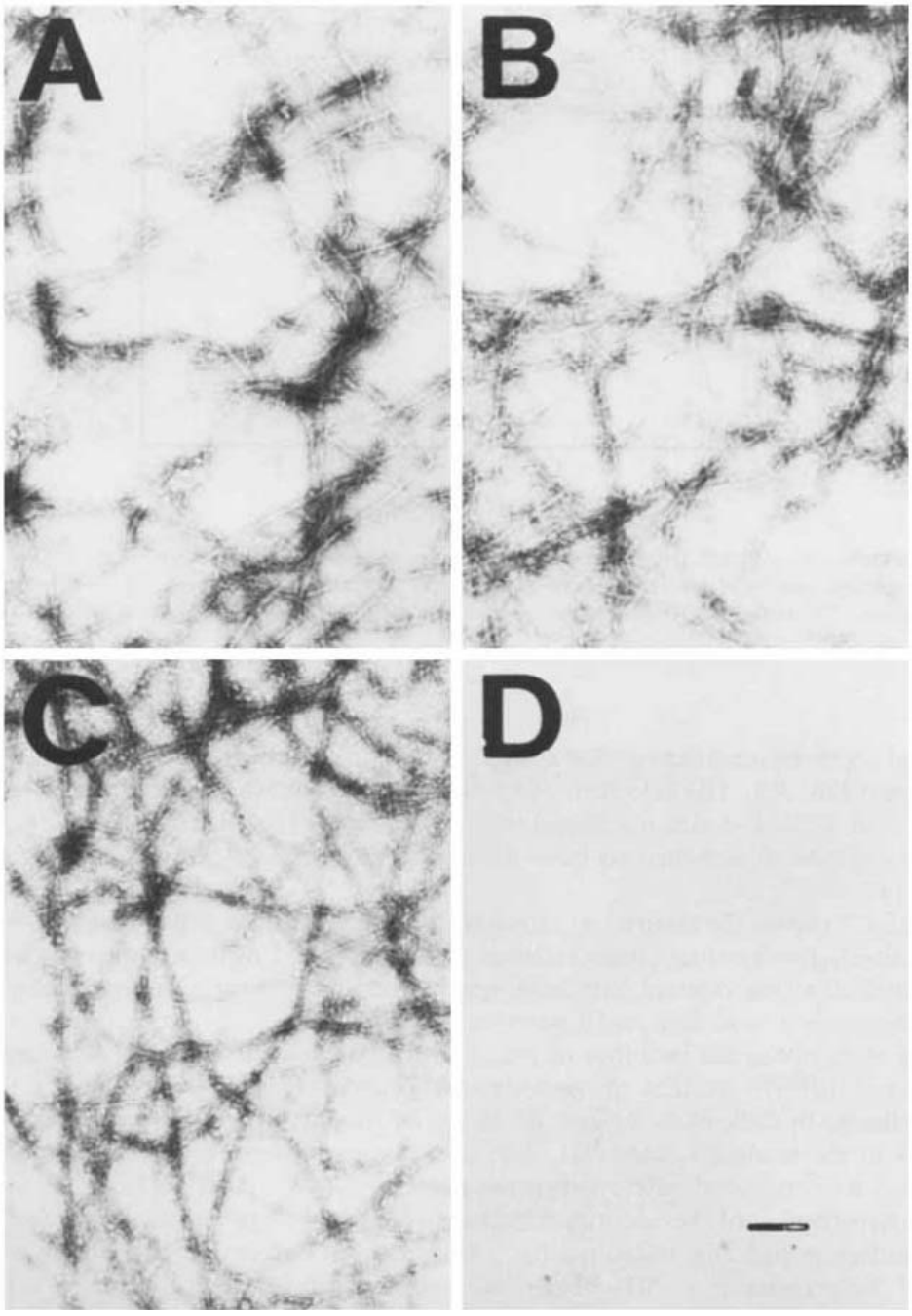

Fig. 5. Morphology of assembled actins from Figure 4. (A) unmodified; (B) [^{14}C]-actin; (C) [^{3}H]-actin; (D) [^{3}H]-actin before addition of ions. Magnification = 59,500×. Bar equals 0.1 μm.

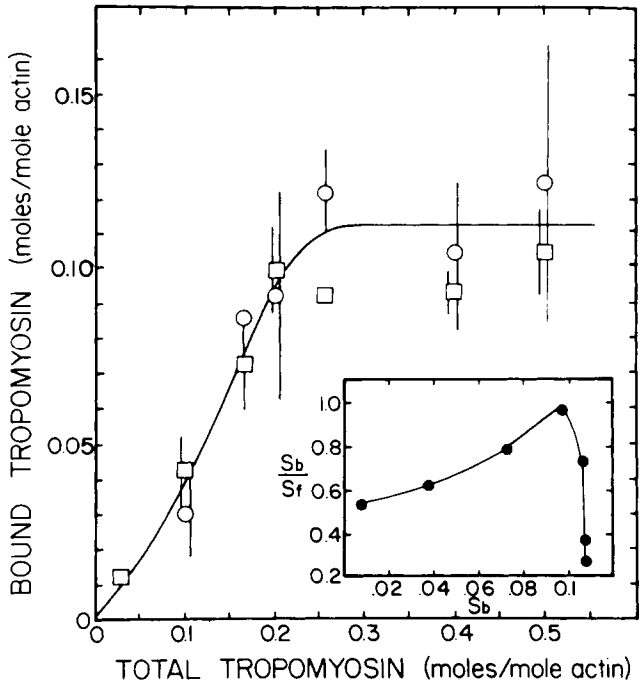

Fig. 6. Binding of tropomyosin and [^{3}H]-tropomyosin to F-actin. Increasing concentrations of tropomyosin were incubated at room temperature with 12 μM F-actin in a buffer containing 10 mM imidazole (pH 7.0), 7 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, 50 nmoles [^{3}H]-tropomyosin (1.2×10^5 dpm/μg) for 16 hr. Total volume of each sample was 1.0 ml. Six aliquots (150 μl each) were pipetted into 240-μl Airfuge tubes; 40 μl of solution was removed from each tube before and after centrifuging at 1.7×10^5 *g* for 10 min. Three aliquots (□) were used to determine decrease in radioactivity after centrifuging, and three aliquots (○) were used to determine the decrease in F-actin and tropomyosin concentrations after centrifuging. Standard deviation (when greater than symbol size) is indicated by error bars. Inset: Scatchard analysis of [^{3}H]-tropomyosin binding to F-actin. The presence of a maximum in this binding analysis is indicative of positive cooperativity as reported for tropomyosin binding to Acanthamoeba actin [37]. S_b = moles of bound tropomyosin; S_f = moles of unbound tropomyosin.

tropomyosin exposed for successively longer times (Fig. 8C) show a large number of other components not visible in the gel stained with Coomassie blue.

DISCUSSION

The method of in vitro labeling a purified protein using reductive methylation is advantageous because the experimenter can control: (a) which protein is being labeled; (b) the specific activity to which it is labeled; and (c) which isotope (^{3}H or ^{14}C) is incorporated. This study indicates that the reductive methylation reaction does not affect the specific protein binding characteristics of cytoskeletal proteins.

The reductive methylation procedure is mild and results in high specific activity when using sodium borohydride of high specific activity (20 Ci/mmole). Most

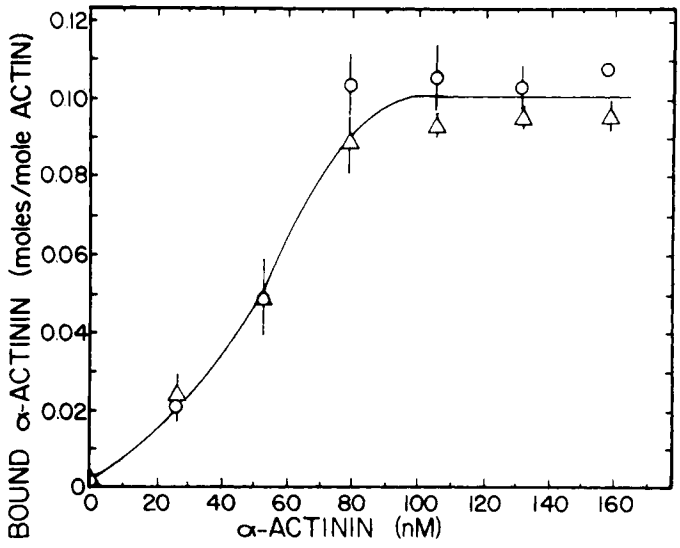

Fig. 7. Binding of α-actinin and [^{3}H]-α-actinin to F-actin at 4°C. Increasing amounts of α-actinin were incubated 3 hr at 4°C in a solution containing 4.8 μM F-actin, 100 mM KCl, 4 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 20 mM Tris-acetate (pH 7.6). Aliquots of each sample were withdrawn before and after centrifugation at 1.7 × 10^5 *g* for 20 min for either radioactivity determination ([^{3}H]-α-actinin) or SDS-PAGE analysis (unlabeled α-actinin). Pellets from [^{3}H]-α-actinin incubations were resuspended and radioactivity contained therein was determined. (△) Binding curve for [^{3}H]-α-actinin average of duplicates from two preparations; (○) binding curve for unlabeled α-actinin, average of duplicates from three preparations. Error bars represent standard deviation.

biochemical techniques do not require greater than 10^5 dpm/μg protein, but if higher specific activities are necessary, a higher ratio of formaldehyde to amino groups may be used. Another alternative is to use sodium borohydride of very high specific activity (60 Ci/mmole, New England Nuclear). While the labeling reaction will not necessarily give the same specific activity for all proteins in a mixture, the high specific activities obtained allow for a rapid visualization of the proteins on electrophoretograms by fluorography. For experiments in which it is necessary to use ultrapure proteins, subjecting a sample to labeling, electrophoresis, and fluorography can be an excellent method for detecting impurities that do not show up by conventional staining methods.

Most of the proteins studied here are normally purified and stored in Tris buffers. Prior to labeling, the Tris buffer must be replaced by a suitable nonreactive buffer. Sodium pyrophosphate at pH 8.5 was found to work well for most of the proteins studied. However, α-actinin precipitated upon exchanging the Tris buffer with either phosphate or pyrophosphate buffer at pH 8.5. The precipitation problem was eliminated by using 0.1 M Bicine (pH 8.0) for the labeling reaction with α-actinin. Use of a higher pH should lead to a more complete labeling of the proteins; at pHs between 8.0 and 8.5 all the proteins tested were stable, but no difference in specific activity was observed for any protein labeled at the extremes of pH in this range.

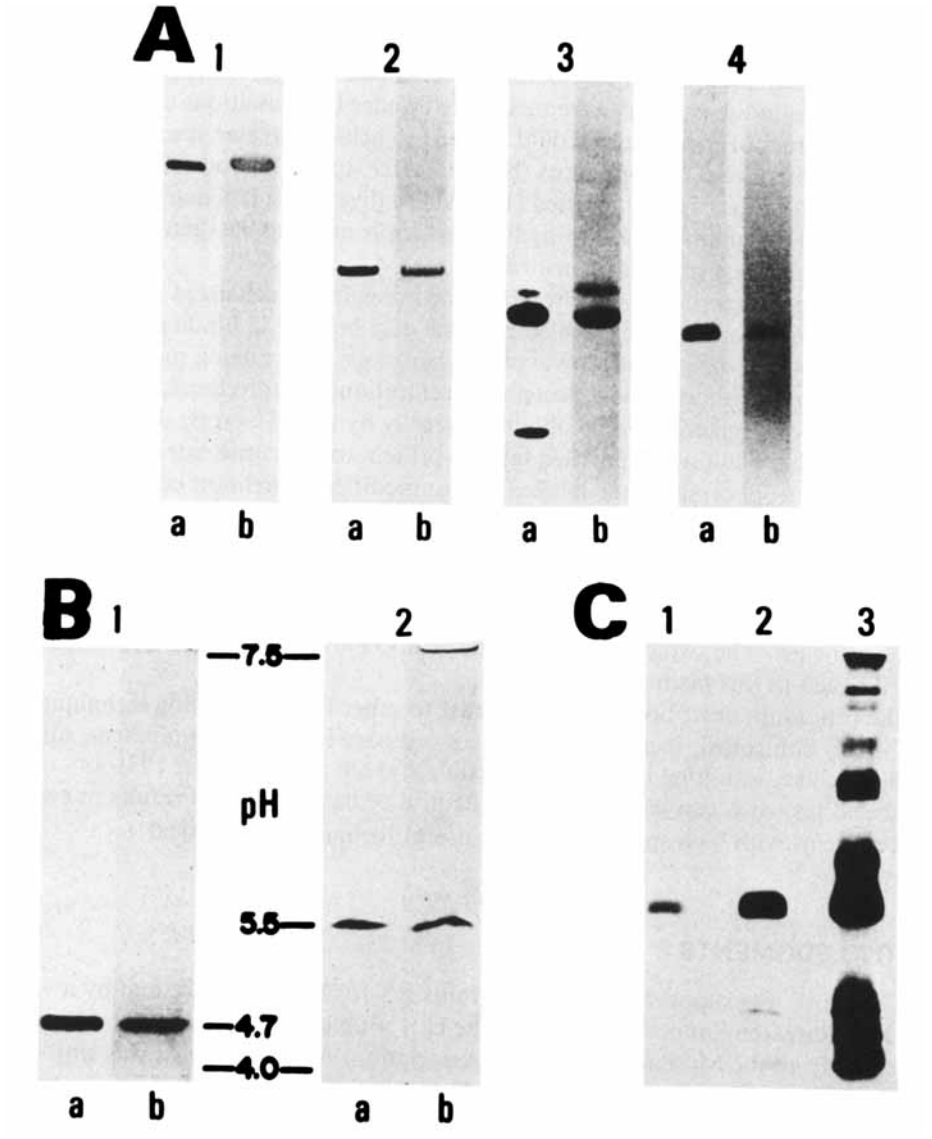

Fig. 8. Polyacrylamide gel electrophoresis and isoelectric focusing of radiolabeled and unmodified cytoskeletal proteins. Gels labeled a are fluorographs of the labeled [^{3}H]-proteins and gels labeled b are the unmodified controls. (A) SDS-PAGE: (1) muscle α-actinin; (2) muscle actin; (3) muscle tropomyosin; (4) brain tropomyosin. (B) Isoelectric focusing gels: (1) rabbit muscle tropomyosin; (2) rabbit muscle actin. (C) Effect of time of fluorographic exposure on visualization of minor components in sample. Scans of SDS-PAGE of this brain tropomyosin sample after Coomassie blue staining shows greater than 97% homogeneity in the two tropomyosin bands. Exposures were (1) 2 hr, (2) 6 hr, (3) 24 hr.

Proteins which are not stable at a pH of 8.0 may be labeled using [^{14}C]-formaldehyde and sodium cyanoborohydride at a pH of 7.0; however, the lower specific activity of the [^{14}C]-formaldehyde (20 mCi/mmole) results in much lower protein specific activity than when [^{3}H]-sodium borohydride is used. Since only 2% of the amino groups in the proteins were methylated under the conditions employed here, higher degrees of methylation could be used to achieve greater specific activities. Under optimal labeling conditions (borate buffer, pH 9.0) about 80% of the available amino groups can be modified [14]. Alternatively, the labeling at pH 7.0 could employ [^{3}H]-sodium cyanoborohydride, which is more stable than sodium borohydride in aqueous solution at neutral pH.

The binding properties of the proteins tested remained unchanged after labeling. Therefore, trace amounts of labeled proteins may be used in binding studies resulting in improved quantitation by avoiding laborious gel scanning methods or inaccurate spectral methods at low protein concentrations. Furthermore, purification yields of each protein can be obtained directly by use of isotope dilution methods following addition of purified labeled protein to the curde extract.

The coelectrophoresis of the labeled and unmodified protein on both SDS gels and denaturing isoelectric focusing gels shows that the method could be applied to identify a protein in a complex mixture of proteins. By adding an amount of a purified, labeled protein (below that detectable by dye binding) to a mixture of proteins, one may electrophorese, stain, and then locate the labeled protein by fluorography, all in the same gel. The two dimensional system of O'Farrell [33] would be particularly well suited to this method.

The procedure described here, in contrast to other in vitro labeling techniques, is mild, easily controlled, inexpensive, involves ordinary laboratory equipment, utilizes radionuclides with long half-lives, is flexible enough to yield either [^{3}H]- or [^{14}C]-labeled proteins, can be performed at neutral or basic pHs, and results in cytoskeletal proteins with high specific activities useful for many biochemical applications.

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