Preparation of Irreversibly Sickled Cell β-Actin from Normal Red Blood Cell β-Actin[†,‡]

Ann Abraham,[§] F. Aladar Bencsath,[§,||] Archil Shartava,[⊥,@] David G. Kakhniashvili,[⊥,@] and Steven R. Goodman*[,⊥,@]

Mass Spectrometry and Protein Structure Laboratory, Department of Biochemistry and Molecular Biology, Department of Cell Biology and Neuroscience, and USA Comprehensive Sickle Cell Center, College of Medicine, University of South Alabama, Mobile, Alabama 36688

Received April 4, 2001; Revised Manuscript Received October 24, 2001

ABSTRACT: We have previously demonstrated that an oxidative change, the formation of a disulfide bridge between two cysteine residues, in the membrane protein β-actin is primarily responsible for locking the irreversibly sickled red blood cells (ISCs) of sickle cell anemic patients into the sickle shape. To support studies on biological and chemical characterization of the oxidized β-actin and pharmacological research toward the reversal of the oxidation, we attempted to prepare oxidized β-actin from normal red blood cell (RBC) β-actin by a chemical reaction, expecting a product equivalent to that found in ISCs. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB, or Ellman's reagent) was used for the oxidation. We proved the absence of accessible sulfhydryl groups in the oxidized product using liquid chromatography (LC) with both UV and fluorescence detection. Polymerization assays indicated that the chemically produced ISC actin demonstrated the same kinetics as ISC actin obtained from patients with sickle cell disease. The effect of the oxidation could be reversed by the use of the reducing agent tris(carboxyethyl)phosphine (TCEP).

Irreversibly sickled cells (ISCs)[1] of sickle cell anemic patients remain sickled even under conditions where they are well-oxygenated and hemoglobin is depolymerized (*1*). Earlier investigations in our laboratories have shown (*2, 3*) that the result of an oxidative change, a disulfide bond between ^{284}Cys and ^{373}Cys of the membrane protein β-actin, distinguishes ISC β-actin from normal RBC β-actin. This single S−S bond not only limits the cell's normal functions but also alters its adhesive characteristics and the fragility of the membrane, causes capillary occlusion and tissue damage, and probably is an important component of vasoocclusive crises. It is reasonable to expect that a properly designed pharmacon could inhibit, and maybe even reverse, the oxidative reaction and ameliorate the disease (*4*).

For experiments aimed at investigating the chemical and functional characteristics of ISC β-actin, a considerable quantity of ISC β-actin is needed. A polymerization experiment, or an association experiment (*2*) with spectrin, β-actin, and protein 4.1, requires at least 0.1 mg of actin. To crystallize actin for X-ray crystallography, one needs several milligrams in a very pure form. We cannot expect the necessary amounts of blood from sickle cell patients, because irreversibly sickled cells make up just a small portion of the RBCs, and large volumes of blood are not available from these already anemic patients. Therefore, we attempted to generate ISC β-actin from normal RBC β-actin using the more readily available normal human blood as an alternative source. During the investigations, efforts were also spent to minimize the amounts of actin that were needed for the experiments. We employed reverse phase LC to purify actin for the redox reactions and chemical tests, and we implemented a fluorescence-based polymerization assay carried out in the microflow cell of the LC fluorescence detector.

EXPERIMENTAL PROCEDURES

Preparation of Normal RBC β-Actin. Normal β-actin was isolated from the blood of healthy adults using our previously published method (*2*), with a modification that replaced size exclusion chromatography with reverse phase liquid chromatography with UV detection (LC/UV). However, in a few instances, the original size exclusion chromatography was used to obtain larger amounts of β-actin. SDS−PAGE indicated homogeneous β-actin.

Briefly, the ghosts obtained from the RBC fraction of 20−30 mL of blood by lysis and centrifugation were treated with a Triton X-100 buffer to remove the lipid layer. The remaining skeletons were dissociated with a Tris buffer. From the resulting mixture of skeletal proteins (spectrin, protein 4.1, and β-actin), β-actin was isolated by LC/UV as follows,

[†] This work was supported by National Institutes of Health Grant 3P60 HL38639 to the USA Comprehensive Sickle Cell Center on which S.R.G. serves as Program Director.

[‡] A part of this work was presented at the 48th Conference on Mass Spectrometry and Allied Topics, June 11−15, 2000, Long Beach, CA.

* To whom all correspondence should be addressed. Present address: Department of Molecular and Cell Biology, The University of Texas at Dallas, Biology F03.1, Box 830688, Richardson, TX 75083-0688. Phone: (972) 883-4872. E-mail: sgoodmn@utdallas.edu.

[§] Mass Spectrometry and Protein Structure Laboratory.

[||] Department of Biochemistry and Molecular Biology.

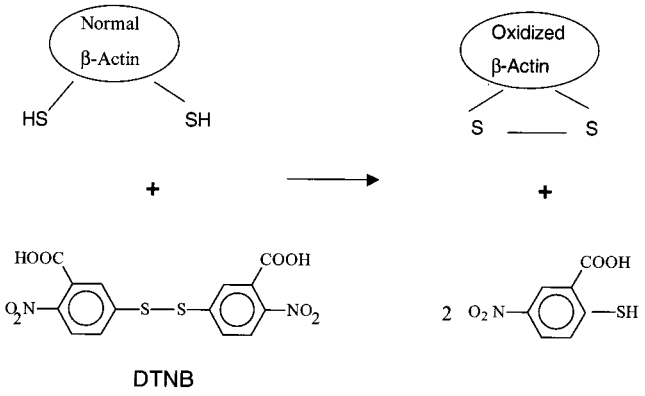
[⊥] Department of Cell Biology and Neuroscience.

[@] USA Comprehensive Sickle Cell Center.

[1] Abbreviations: ISC, irreversibly sickled cell; DTNB (Ellman's reagent), 5,5′-dithiobis(2-nitrobenzoic acid); FAB, fast atom bombardment ionization; FD, fluorescence detection; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MBrB, monobromobimane; MS, mass spectrometry; RBC, red blood cell; UV, ultraviolet photometry; TCEP, tris(carboxyethyl)phosphine; TFA, trifluoroacetic acid.

10.1021/bi010685v CCC: $22.00
Published on Web 12/08/2001

Scheme 1: Oxidation of Normal RBC β-Actin



or by size exclusion chromatography using a Sepharose 4B gel filtration column (*2*) (yield of ~1 mg of β-actin).

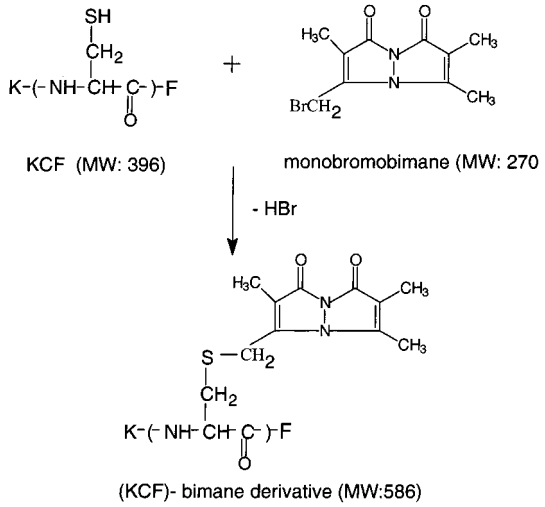
LC/UV Conditions for Protein Separation. We used a Waters 600-MS system equipped with a Waters 484MS absorbance detector (Waters, Milford, MA) and a Linear Instruments model LC305 fluorescence detector (model FL-503, Chrom Tech, Inc., Apple Valley, MN). A Jupiter 5 μm C4, 300 Å, 250 mm × 4.6 mm analytical column (Phenomenex, Torrance, CA) was used with a gradient program of 50 to 80% B over the course of 60 min and a flow rate of 1 mL/min. Eluent A was 0.1% TFA in water, and eluent B was 0.1% TFA in 80% aqueous acetonitrile. A maximum of a 100 μL (240 μg) protein sample could be injected without risking separation efficiency. The proteins eluting across the chromatographic peaks were manually collected (four or five injections), pooled, and freeze-dried in 1.6 mL polypropylene tubes.

MS Identification of the Isolated β-Actin. Portions of the dried isolates were analyzed with a MALDI-TOF mass spectrometer using the Voyager Elite instrument with delayed extraction technology in the linear mode (PerSeptive Biosystems, Framingham, MA) at the Pharmacology Department, University of Alabama at Birmingham (Birmingham, AL). The acceleration voltage was set to 25 kV, and 10−50 laser shots were summed for the spectra. Sinapinic acid (Aldrich, Milwaukee, WI) dissolved in acetonitrile and 0.1% TFA (1:1) was used as a matrix. The mass spectrometer was calibrated with bovine serum albumin (BSA). The protein isolates, in the 1.6 mL tubes, were reconstituted with 20 μL of matrix, and 1 μL portions were pipetted onto the mass spectrometer's smooth plate sampler.

Oxidation of β-Actin (Scheme 1). One milliliter of a β-actin solution (0.160 mg/mL, ~3.8 nmol) was reacted with 6 μL of DTNB (30 μg, 76 nmol; 20-fold excess) in 1% $NaHCO_3$ at 4 °C for 18 h. The solution was then dialyzed for 48 h against a dialysis buffer containing 2 mM Tris, 0.4 mM ATP, and 0.5 mM NaN_3 (pH 7.8) that was changed every 12 h. After dialysis, the solution was concentrated to ~800 μL.

Reduction of Oxidized β-Actin: Regeneration of Sulfhydryl Groups. Oxidized β-actin was reduced with tris(2-carboxyethyl)phosphine (TCEP) at 4 °C for 18 h. To 250 μL of oxidized β-actin (50 μg, 1.2 nmol) was added 8 μL of an aqueous TCEP solution (3 mg/mL, 24 μg), and the mixture was allowed to react overnight at 4 °C. The reduced actin was then reacted with the monobromobimane (MBrB) thiol reagent (*5*, *6*) in an identical process that is described next for the normal actin and analyzed by LC/UV/FD to demonstrate the presence of the regenerated sulfhydryl groups.

Scheme 2: Fluorescent Labeling of Sulfhydryl Groups Exemplified by the Reaction of the Tryptic Peptide KCF with Monobromobimane



Derivatization of β-Actin and Related Peptides with the Monobromobimane Fluorescent Reagent (Scheme 2). β-Actin samples (normal, oxidized, and regenerated) containing ~100 μg of protein (~2.3 nmol) were reacted with a 26-fold excess of the monobromobimane reagent (4 μL of MBrB, 4 mg/mL, 60 nmol in an acetonitrile solution) at pH 7.6 and 4 °C for 20 h. The reaction was stopped by the addition of one drop of acetic acid. The reaction mixture was analyzed by LC using two detectors; one injection was made with UV and another with fluorescence detection (LC/UV/FD).

A similar derivatization and LC procedure was employed to generate fluorescent derivatives from the two peptides CDVDIR and KCF that contained the important ^{284}Cys and ^{373}Cys residues. These bimane-labeled model peptides were prepared to help identify the fluorescent peptides observed in the peptide map of the bimane-labeled β-actin. The peptides themselves were synthesized in the course of our earlier work to help locate the corresponding tryptic peptides in the β-actin peptide map (*2*).

The derivatization reaction with these relatively low MW peptides (719 and 396, respectively) requires only a few minutes for completion. The identities of the bimane-labeled peptides were confirmed by fast atom bombardment mass spectrometry (FAB/MS).

LC/UV/FD Conditions for Bimane-Derivatized β-Actin. A 250 mm × 4.6 mm, RP-Protein-C4 300 Å (Vydac, Hesperia, CA) column was used with the following gradient program: from 20 to 50% solvent B over the course of 20 min and then from 50 to 85% B over the course of 40 min with a flow rate of 1 mL/min. Eluents A and B were identical to those used for protein separation. The UV detector was used at 210 nm. The fluorescence detector's (FD's) excitation wavelength was set to 398 nm and that of emission to 480 nm.

FAB/MS of the Bimane-Labeled Peptides. A VG70-250 SEQ instrument equipped with a saddle-field FAB gun was used. Four microliters of the freshly derivatized mixtures containing ~4 μg of peptides was mixed with 1 μL of glycerol on the flat surface of the probe tip. The glycerol

served as the FAB matrix. Scanning spectra were acquired in the mass range of *m*/*z* 100−1200 at a speed of 5 s/decade and a mass resolution of 1500.

Generation of Tryptic Maps from Normal β-Actin and Oxidized β-Actin after the Reaction with Monobromobimane. Normal β-actin and oxidized β-actin, ∼100 μg (2.3 nmol) each, after the reaction with MBrB and isolation by LC/UV, were concentrated to ∼1800 μL, neutralized by the addition of 200 μL of 8% NH_4HCO_3 (pH 7.6), and digested with 120 μL of trypsin (3 mg/mL) for 18 h at 37 °C. The digests were analyzed by LC/FD.

LC/FD Conditions for the Tryptic Digest (Peptide Map). A Luna 5 μm, C18, 150 mm × 4.6 mm RP column was used (Phenomenex). Digests (300 μL) were injected during the experiments. Eluent A (0.1% TFA in water) and eluent B (0.1% TFA in 80% aqueous acetonitrile) were used with a linear gradient of 0 to 100% B over the course of 100 min, and a flow rate of 1 mL/min. The FD wavelength settings were as follows: 398 nm for excitation and 480 nm for emission.

Polymerization Assay with Normal and Oxidized β-Actin. A micropolymerization assay was developed for testing actin functionality by using the LC fluorimetric detector. The volume of the fluorimeter's analytical cell was 8 μL. The original input tubing (FEP TUB, 0.010 in. × $^1/_{16}$ in., Upchurch Scientific, Oak Harbor, WA) at the bottom entry of the cell was shortened to ∼4 in., and connected with a $^1/_{16}$ in. PEEK union (Upchurch) to 1.5 in. long, 0.020 in. × $^1/_{16}$ in. PEEK tubing (Alltech Associates, Deerfield, IL). The latter served as the injection port; the 0.020 in. inside diameter allowed an airtight injection using a 50 μL syringe (800 series, needle gauge 26s, point style 3; Hamilton Company, Reno, NV). The input tubing and the syringe were held horizontally, slightly below the level of the cell. The syringe was kept in the tubing after the injection of the β-actin test solution until the end of the experiment (40−60 min).

The assay is a copolymerization experiment (*7*, *8*); the fluorescence of the β-actin mixed with pyrene-labeled α-actin (P-actin from Cytoskeleton, Denver, CO) in a ratio of 9:1 in a polymerizing buffer was followed during the polymerization process (excitation at 365 nm, emission at 407 nm).

The actin and buffer solutions and their mixtures were kept in an ice bath until injection. The normal β-actin solution was diluted with 0.5 mM mercaptoethanol to a final actin concentration of 0.3 mg/mL, and mixed with a freshly prepared 5 mg/mL P-actin solution so that a 10:1 β-actin:P-actin ratio could be reached. A 40 μL aliquot of this mixture was injected into the flow cell, and the fluorimeter was zeroed. Then to 135 μL of the actin mixture was added 15 μL of polymerization buffer containing 500 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP, and this buffer was mixed with the syringe contents (final β-actin concentration of 0.266 mg/mL); a 40−60 μL portion of the mixed solution was injected into the flow cell. The fluorescence measurement started immediately after injection, by recording fluorescence with an integrating recorder (HP 3390A) and by manually writing the readings in every minute at the beginning, and then every 2−4 min after the first half-hour.

The polymerization experiments with the different kinds of actin samples were carried out in duplicate (P-actin and oxidized β-actin) or triplicate (normal β-actin). The time−

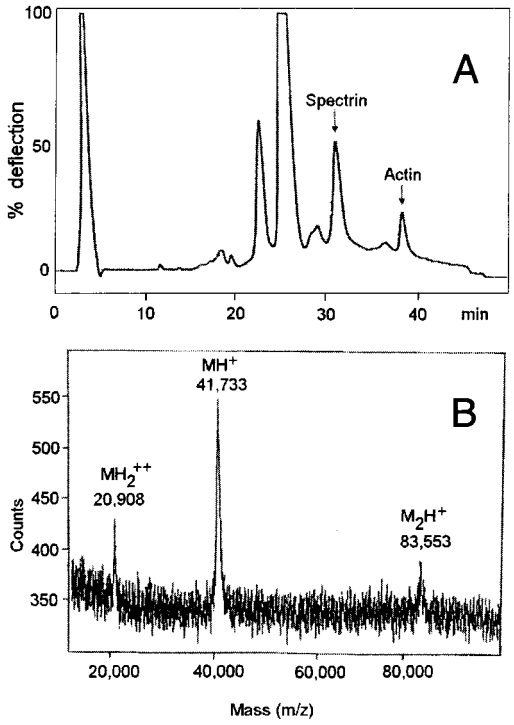


FIGURE 1: (A) LC trace of normal RBC β-actin (t_R = 39 min) separation from a dissociated skeletal protein mixture and (B) MALDI-TOF mass spectrum of the actin isolate.

fluorescence intensity figures obtained in each experiment were entered into a Microsoft Excel (Microsoft Corp., Seattle, WA) worksheet, and these raw data were adjusted by an increment that brought the first reading to 0 min, 0.000 fluorescence. The adjusted data were transferred into a SigmaPlot (SigmaPlot for Windows, version 4.0, SPSS Science, Chicago, IL) worksheet to create a fluorescence versus polymerization time scatter plot. A consecutive nonlinear regression analysis produced a fitting curve for the data points in the scatter plot by using the sigmoidal Chapman equation in a modified three-parameter form:

$$y = y(0) + a(1 - e^{-bx})$$

where *y* is the fluorescence (in fluorescence units) recorded at time *x* (minutes), *a* is the maximum fluorescence at the end of the experiment, and *b* is the polymerization rate constant, the main result of the plotting and regression analysis process. The equation and its use were identical to those employed in our earlier work (*9*), with the difference being that minutes were used for time measurements instead of seconds.

RESULTS AND DISCUSSION

β-Actin is traditionally separated from the other erythrocyte membrane skeletal proteins by size exclusion chromatography on a gel filtration column, which is a daylong process. The dialysis step, necessitated by the high concentration of buffer salt (2 M tris) used for the chromatography, further extends the isolation process. The Jupiter C4 column with the relatively volatile 0.1% TFA in the eluent successfully separated actin from the other components; actin was the latest eluting protein (t_R = 39 min, Figure 1A). We found that maximum amount of 240 μg of total protein could be

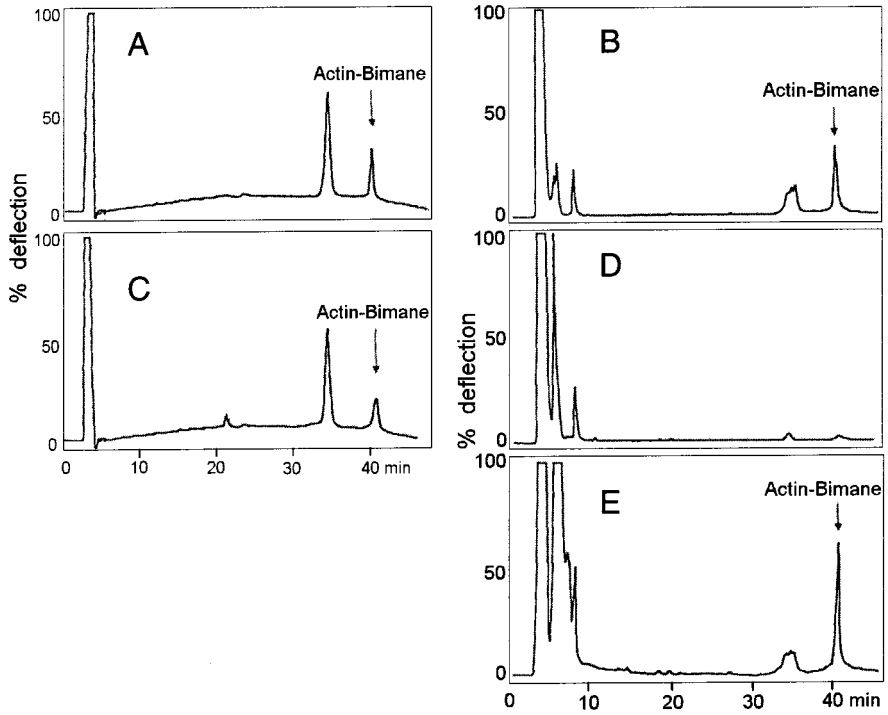


FIGURE 2: LC/UV and LC/FD traces of bromobimane-treated normal and oxidized β-actin: (A and B) normal β-actin (UV and FD traces, respectively), (C and D) oxidized β-actin (UV and FD traces, respectively), and (E) oxidized β-actin, reduced with TCEP prior to bromobimane treatment (FD trace).

injected, without overloading this 4.6 mm diameter analytical column. A 10 mm diameter semipreparative column would probably increase the productivity by a factor of ~4.

The isolated components eluted across the chromatographic peaks were submitted to MALDI-TOF MS analysis, and the resulting spectrum proved the identity of the peak that eluted at 39 min (Figure 1B). The dominant ion at *m*/*z* 41 733 represented the molecular ion of β-actin, and its purity was confirmed by the twice-protonated molecular mass of *m*/*z* 20 908 as well as the molecular ion cluster that appears with a lower intensity at *m*/*z* 85 553.

Once separated, it was necessary to prove the presence of the intact sulfhydryl groups at ^{284}Cys and ^{373}Cys. Therefore, the isolated actin was derivatized with the fluorescent sulfhydryl reagent monobromobimane, and the reaction mixture was subjected to two chromatographic analyses, using UV and FD, one at a time. Normal β-actin appeared with identical t_Rs in both the UV and FD chromatograms (top panels of Figure 2A,B). After oxidation, however, only the UV chromatogram exhibited the actin's elution (somewhat reduced in size; Figure 2C), while in the FD chromatogram, only traces appeared, indicating the lack of accessible thiol groups (Figure 2D).

TCEP addition to the oxidized β-actin seems to reverse the oxidation, and the regenerated thiol groups are clearly visible (Figure 2E). It is to be noticed that the TCEP-reduced actin seems to have increased in fluorescence compared to the normal unoxidized actin in Figure 2B, indicating that a part of the thiol population in the normal actin is in the oxidized state.

The positions of the fluorescence labels in the derivatized β-actin were determined by peptide mapping. The chromatogram obtained from normal actin after the tryptic digest displays among several intense peaks two dominant ones at t_Rs of 29.4 and 30.5 min (Figure 3A, top chromatogram). These peaks are absent from the peptide map generated from the oxidized β-actin (Figure 3B). Because of their retention times match exactly, the fluorescence-labeled synthetic CDVDIR and KCF model peptides have properly identified these peaks (Figure 3C).

The FAB mass spectra below the HPLC traces (Figure 3D,E) confirm the identities of the derivatized synthetic peptides; the dominant peaks at *m*/*z* 910 and 587 represent the protonated molecular ions of the CDVDIR and KCF derivatives, respectively. The sodium adduct ions appear in higher abundances and larger numbers in the CDVDIR spectrum (*m*/*z* 932, 954, and 976) than in the KCF spectrum (*m*/*z* 619) because of the presence of the alkali-phil acidic aspartate residues (two Ds) in the former peptide.

One of the most interesting and physiologically important molecular functions of β-actin is polymerization. In earlier experiments (*9*), we used light scattering measurements in a Hitachi F-2000 spectrofluorimeter's 100 μL cuvette that required 100 μL of an actin solution (~40 μg of actin). The fluorescent assay we adopted for the work presented here requires lower actin concentrations (0.15−0.2 mg/mL) and a smaller volume. Even as we generously flooded the 8 μL flow cell with 60 μL actin solutions to fill it bubble-free, we used no more than 16 μg of actin. With careful manipulation, this amount could easily be cut in half.

The graphs in Figure 4 depict the polymerization processes in three individual experiments typical of normal β-actin (▲), oxidized β-actin (●), and P-actin alone [0.027 mg/mL, control experiment (○)]. The corresponding polymerization rates were computed as 0.140 min^{-1} for normal β-actin and 0.079 min^{-1} for oxidized β-actin [average rates of 0.155 (n = 3) and 0.066 min^{-1} (n = 2), respectively]. These figures are good approximations of those obtained earlier in our laboratory for normal and ISC β-actin (*9*), when light scattering was used to follow the polymerization process (1.6

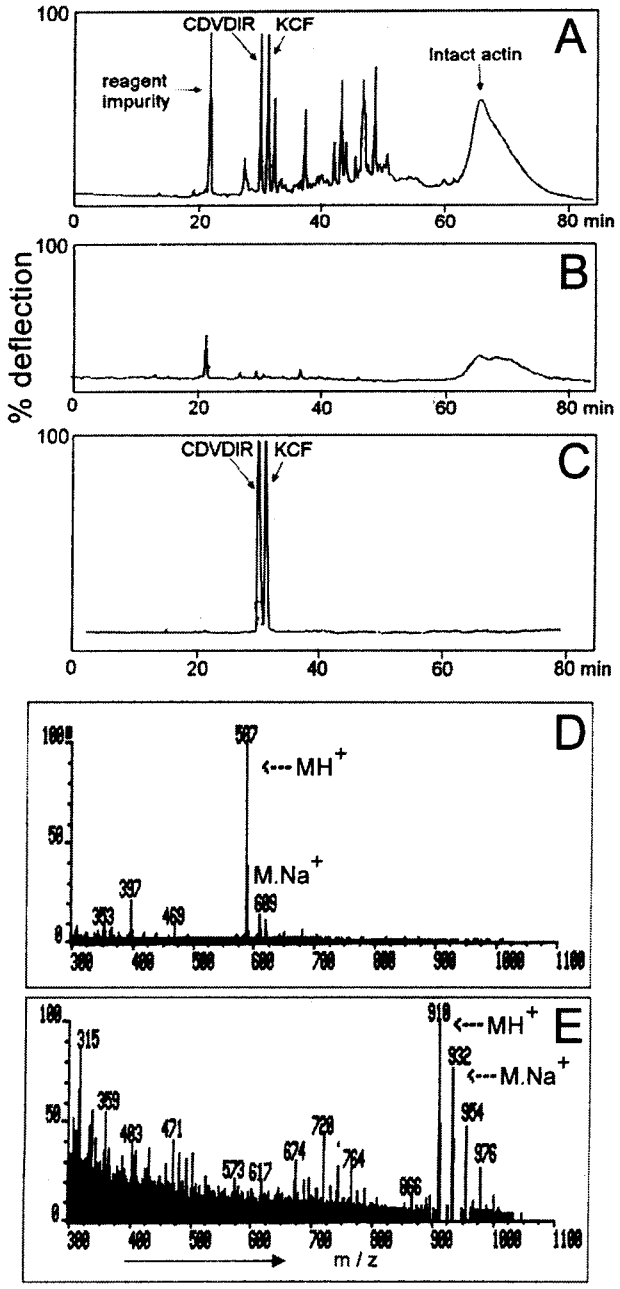


FIGURE 3: Tryptic maps of bromobimane-treated normal β-actin (A) and oxidized β-actin (B), and the corresponding chromatogram of the mixture of the bromobimane-treated model peptides CDVDIR and KCF (C) with t_Rs of 29.4 and 30.5 min, respectively. The FAB mass spectra below are those of the CDVDIR and KCF bimane derivatives.

$\times 10^{-3}$ s^{-1} = 0.096 min^{-1} for normal β-actin and 1.1 $\times$ 10^{-3} s^{-1} = 0.066 min^{-1} for ISC β-actin). The low-intensity final fluorescence value observed in the control experiment (Figure 4) is indirect evidence of successful copolymerizations in the mixed actin experiments.

From the results discussed above, we conclude that β-actin isolated from normal human blood can be converted by oxidation with Ellman's reagent into a product, which is apparently identical to ISC β-actin according to its chromatographic behavior (retention time), chemical characteristics (no sulfhydryl groups are available for the fluorescent labeling reagent), and polymerization kinetics. The reaction should allow production of chemically prepared ISC β-actin from normal RBC β-actin in the desired large quantities. The oxidative reaction was reversed by the reducing agent TCEP; that is, normal β-actin was generated from chemically prepared ISC β-actin. This in vitro reaction justifies our hope for a future pharmacological intervention of the oxidative damage leading to the formation of ISCs and dense cells.

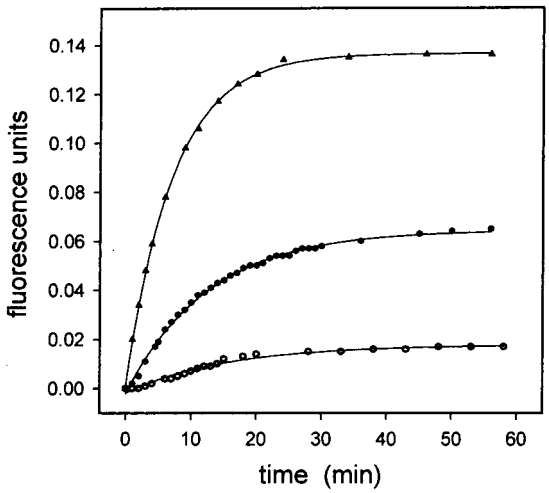


FIGURE 4: Polymerization kinetics of β-actin (▲) and oxidized β-actin (●) with P-actin, and P-actin alone (○). The concentration of β-actin, both normal and oxidized, was 0.266 mg/mL (6.2 μM). The concentration of P-actin was 0.027 mg/mL (0.62 μM) in each experiment. The solutions also contained 50 mM KCl, 2 mM $MgCl_2$, and 1 mM ATP. The data points in the plots correspond to the fluorescence−time data pairs recorded during the polymerization, and the connecting curves are the results of the nonlinear regression analyses (see the details in the text).

ACKNOWLEDGMENT

We acknowledge the friendly help of Dr. Marion Kirk and Dr. Lori Coward (University of Alabama at Birmingham) for providing us with the MALDI spectra of the LC isolates. We also thank Dr. Ashley Davis, of Cytoskeleton, for providing us with references and guidance in the use of the P-actin for the micropolymerization assay and Elizabeth Thomas (Department of Surgery, University of Al Ain, Al Ain, United Arab Emirates) for helping us in the graphical presentation of the experimental data.

REFERENCES

1. Kaul, D. K., Fabry, M. E., Windish, P., Baez, S., and Nagel, R. L. (1983) *J. Clin. Invest. 72*, 22−31.
2. Shartava, A., Monteiro, C. A., Bencsath, F. A., Schneider, K., Chait, B. T., Gussio, R., Casoria-Scott, L. A., Shah, A. K., Heuerman, C. A., and Goodman, S. R. (1995) *J. Cell Biol. 128* (5), 805−818.
3. Bencsath, F. A., Shartava, A., Monteiro, C. A., and Goodman, S. R. (1996) *Biochemistry 35*, 4403−4408.
4. Gibson, X. A., Shartava, A., McIntyre, J., Monteiro, C. A., Zhang, Y., Shah, A., Campbell, N. F., and Goodman, S. R. (1998) *Blood 9* (11), 4373−4378.
5. Kosower, E. M., and Kosower, N. S. (1995) *Methods Enzymol. 251* (12), 133−166.
6. Fahey, R. C., Newton, G. L., Dorian, R., and Kosower, E. M. (1981*) Anal. Biochem. 111*, 357−365.
7. Kouyama, T., and Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33−38.
8. Cooper, J. A., Walker, S. B., and Pollard, T. B. (1983) *J. Muscle Res. Cell Motil. 4*, 253−262.
9. Shartava, A., Kern, W., Shah, A. K., and Goodman, S. R. (1997) *Am. J. Hematol. 55*, 97−103.

BI010685V