Identification of the Disulfide-Linked Peptide in Irreversibly Sickled Cell β-Actin

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ABSTRACT: We have previously demonstrated that the membrane skeletons of irreversibly sickled cells (ISCs) dissociate more slowly at 37 °C, in high ionic strength Triton X-100 buffer, than do the membrane skeletons of reversibly sickled cells or control erythrocytes [Shartava et al. (1995) *J. Cell. Biol. 128*, 805−818]. Furthermore, we demonstrated that the major cause of this slow dissociation was a single posttranslational modification in ISC β-actin. Two sulfhydryl groups (Cys^{284} and Cys^{373}) became inaccessible to thiol reagents because of this modification. We suggested the possibility that the modification was a disulfide bridge between Cys^{284} and Cys^{373} since the reducing agent dithiothreitol restored the sulfhydryl groups. In this article, we directly demonstrate the existence of the disulfide bridge between $cysteine^{284}$ and $cysteine^{373}$ in ISC β-actin. We synthesized the associated ISC β-actin tryptic cystine-peptide (KCF-CDVDIR), characterized it by HPLC, MS, and MSMS, and identified it in the tryptic digest of the ISC β-actin. These results support our earlier suggestion that the oxidative change in ISC β-actin is a major cause of the irreversible sickling phenomenon.

The molecular events which occur within RBCs[1] from homozygous sickle cell patients, and to the environments of the cells, and lead to the sickle cell crisis, organ damage, and mortality are of great interest to the clinical and scientific community [for reviews see Hebbel (1990, 1991), Powers (1990), Francis and Johnson (1991), and Joiner (1993)]. During the course of vasoocclusion, the highest density class of RBCs are selectively trapped in the microvasculature (Kaul et al., 1986, 1989). Approximately 60%−85% of the cells in this high-density class are ISCs which retain the sickled shape in well-oxygenated blood even though the hemoglobin has depolymerized to its tetrameric HbS form (Kaul et al., 1983). The dense, elongated, and nonflexible ISCs appear to block the narrowed lumen of vessels lined primarily with the more adherent lower density reversibly sickled cells (RSCs), and sometimes block by direct capillary occlusion (Kaul et al., 1985; Fabry et al., 1992). For these reasons the ISC is thought to be a major factor in causing the painful sickle cell episodes which are a hallmark of the disease.

The red blood cell contains a membrane skeleton on the cytoplasmic surface of its plasma membrane, composed of spectrin tetramers, protein 4.1, and actin protofilaments (as well as other accessory proteins), which is responsible for maintenance of its biconcave shape and its properties of elasticity and flexibility (Goodman et al., 1988). The first indication that the membrane skeleton was involved in the phenomena leading to the ISC was a demonstration by Lux and colleagues that triton shells (membrane skeletons) prepared from ISCs retained a sickled shape (Lux et al., 1976). We have recently demonstrated that a posttranslational modification of the membrane skeletal protein β-actin leads to slow dissociation of the ISC membrane skeletal components at 37 °C and, therefore, to the inability of the ISC to remodel (Shartava et al., 1995). In these studies we demonstrated that $cysteine^{284}$ and $cysteine^{373}$ were available to labeling, by thiol-reactive reagents, in nonreduced samples of control β-actin but were not accessible in nonreduced ISC β-actin. However, these cysteines could be labeled if the ISC β-actin was first treated with a reducing agent. Furthermore we demonstrated by MALDI mass spectrometry that control and ISC β-actin had indistinguishable molecular weights within the error of measurement ($\pm$100 Da). We therefore suggested that ISC β-actin had a disulfide bridge formed between $cysteine^{284}$ and $cysteine^{373}$. In the current studies we synthesized the relevant cystine-containing peptide complex and identified the KCF-CDVDIR peptide in the tryptic digest of ISC β-actin by mass spectrometry. Since the KCF peptide contains $cysteine^{373}$ and CDVDIR contains $cysteine^{284}$, these results conclusively demonstrated the formation of a $cysteine^{284}$−$cysteine^{373}$ disulfide bridge in ISC β-actin.

EXPERIMENTAL PROCEDURES

Reagents and Synthetic Processes. Formic acid, dithiothreitol (DTT), trypsin (TPCK treated) were purchased from Sigma Chemical Company (St. Louis, MO). 2,4-Dinitro-

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[1] Abbreviations: CF-FAB, continuous-flow FAB; DNPS-Cl, 2,4-dinitrophenylsulfenyl chloride; DNPT-, 2,4-dinitrophenylthio-; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FAB, fast atom bombardment ionization; FMOC, fluorenylmethoxycarbonyl; ISC, irreversibly sickled cell; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; MSMS, tandem mass spectrometry; RBC, red blood cell; RSC, reversibly sickled cell; TFA, trifluoroacetic acid.

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phenylsulfenyl chloride (DNPS-Cl) was from Aldrich (Milwaukee, MI), toluene (ultrapure) was from Alfa Products (Ward Hill, MA), and tetrahydrofurane (HPLC grade) was from J. T. Baker (Phillipsburg, NJ).

KCF and CDVDIR peptides, representing tryptic fragments of actin, were produced by solid phase synthesis using FMOC chemistry on a model 431a peptide synthesizer (Applied Biosystems, Foster City, CA). The identities of the peptides were ascertained by FAB-MS after purifying them by reverse phase HPLC (System Gold, Beckman Instruments, Palo Alto, CA) using a standard 0.1% TFA and 80% acetonitrile with 0.1% TFA gradient.

KCF-CDVDIR disulfide bridge complex was synthesized in two steps using arylsulfenyl halide chemistry (Fontana et al., 1968a), following a previously described protocol (Drewes et al., 1990) with the necessary modifications for low-milligram batch size. Briefly, one of the peptides (KCF) was activated in the first step: KCF, 4 mg (10 μmol), was dissolved in 100 μL of formic acid placed in a 5 mL conical centrifuge tube. DNPS-Cl, 8 mg (34 μmol), dissolved in 300 μL of formic acid was added into the tube while gently shaking and occasionally sonicating. After a 1 h reaction period, 200 μL of tetrahydrofurane and 600 μL of toluene were added, followed by an additional 2 mL of toluene. The reaction mixture was shaken by hand and then centrifuged for 5 min at 2500 rpm. The toluene/tetrahydrofurane phase above the oily sediment was discarded, and the residue was dissolved in 60 μL of tetrahydrofurane. Toluene (500 μL) was added while shaking the vial. After centrifugation, the organic solvent layer was discarded, and the precipitate was dried in air (5.0 mg, 83% yield). Before use, the residue was dissolved in 500 μL of methanol (10 μg/μL). Mass spectra were generated from 1 μL methanolic solution using a glycerol matrix (MW 594).

The second step of the synthetic procedure was the coupling of the activated peptide (DNPT-KCF) to the second peptide, CDVDIR: 1 mg of CDVDIR (MW 566) was dissolved in a 2 mL glass vial by 100 μL of 50 mM NH_4HCO_3 buffer (10 μg/μL), pH = 8.0. 1 μL of this solution was added onto the FAB probe tip, which was holding 3 μL of glycerol. After the mass spectra were recorded, 1 μL of solution of the activated KCF was added onto the probe tip and carefully mixed with the CDVDIR-containing matrix. The disulfide-linked KCF-CDVDIR peptide forms immediately, and the development of the yellow color signals the liberation of the dinitrothiophenolate. The entire 1 mg of CDVDIR in the vial was similarly transformed by adding the proper amount of activated KCF solution (110 μL) to it. Judging from the rapid change of color, the reaction was complete within the first minute. Five minutes later, the reaction mixture was acidified with 4 μL of glacial acetic acid (pH $\approx$ 4). In acidic solution, refrigerated at 4 °C, the peptide was stable for months.

ISC β-actin was prepared from the high-density fraction of RBCs obtained from sickle cell anemia patients, as described previously in detail (Shartava et al., 1995). ISC actin was stored at 4 °C in a concentration of 150−200 μg/mL and used within 48 h of isolation.

Tryptic Digest and Reverse Phase HPLC. Peptide Map Generation. ISC β-actin (200 μg in 1 mL) was incubated with trypsin (50:1 actin: trypsin w/w ratio) in 75 mM NH_4HCO_3, 0.1 mM $CaCl_2$ at pH 7.8 for 20 h at 37 °C. Digested actin was dried to a powder in a Speed-Vac instrument (Savant Instrument, Inc., Farmingdale, NY) and was then resuspended in half of the original volume with HPLC buffer A (0.1% aqueous TFA). The actin digest was loaded onto an ODS 5 μ C_{18} reverse phase column (4.6 mm × 15 cm) with precolumn and eluted using the Beckman System Gold HPLC. The following program was used: 5 min isocratic period with buffer A, followed by a gradient of 0%−100% buffer B (0.1% TFA, 80% ACN) over 90 min. The flow rate was 1 mL/min, and OD_{215} was monitored. Fractions (1 mL) were collected and dried in the Speed-Vac prior to mass spectrometry (MS).

Mass Spectrometry. FAB-MS. A VG 70−250 SEQ hybrid tandem instrument equipped with a saddle-field FAB gun and a continuous-flow fast atom bombardment (CF-FAB) probe was used for the MS analyses. The probe was modified and FAB-MS was conducted as described previously (Shartava et al., 1995).

Tandem Mass Spectrometry. MSMS spectra were obtained as previously described (Shartava et al., 1995). The analyzer quadrupole was scanning from *m*/*z* 200 to *m*/*z* 1200 in 10 s and the MS/MS spectra were recorded in MCA (multiple-channel analyzer) format: five or six continuum spectra at the elution maximum of the sample were summed, and then the resulting spectrum processed (smoothed, peak-detected, and mass converted) in the usual, mass vs relative abundance, bar diagram format.

RESULTS

Synthetic Disulfide-Linked Peptide KCF-CDVDIR. In order to prove the existence of the disulfide bond in ISC β-actin one has to demonstrate the presence of the correlated cystine-peptide KCF-CDVDIR in the tryptic digest. To accomplish this task, first we synthesized the peptide, and then we determined its chromatographic and mass spectrometric (MS) characteristics and used these data for detection and identification.

The synthesis is based on the reaction of arylsulfenyl chloride with cysteine, which was developed as a peptide/protein modifying and cysteine residue protecting tool (Scoffone et al., 1968; Fontana et al., 1968a,b). Arylsulfenyl halides such as 2-nitrophenyl or 2,4-dinitrosulfenyl chloride react with the sulfhydryl groups of peptides and proteins in acetic or formic acid solvent, producing an asymmetric disulfide compound. The asymmetric aryl−alkyl disulfide is stable under moderately acidic conditions but gradually decomposes in alkaline media (Fontana et al., 1968a). However, in the presence of another aliphatic sulfhydryl compound in the alkaline medium, a rapid exchange occurs: the dinitrophenyl moiety in the activated compound is replaced by the other aliphatic sulfhydryl compound. This reaction can be utilized to build disulfide-linked (asymmetric cystine-containing) peptides. Acetic acid and formic acid are excellent solvents for most peptides and many proteins, therefore cysteine containing peptides (such as KCF) can readily be activated by arylsulfenyl halides into a reactive intermediate (*S*-dinitrophenylthio-KCF). The reactive intermediate, as mentioned above, easily reacts with a second cysteine peptide in mildly alkaline medium (50 mM NH_4HCO_3, pH $\approx$ 8.0) producing the desired disulfide linked peptide (KCF-CDVDIR). With the production of the S−S-linked peptide, an equivalent amount of dinitrophenolate anion is liberated, and the progress of the reaction may be

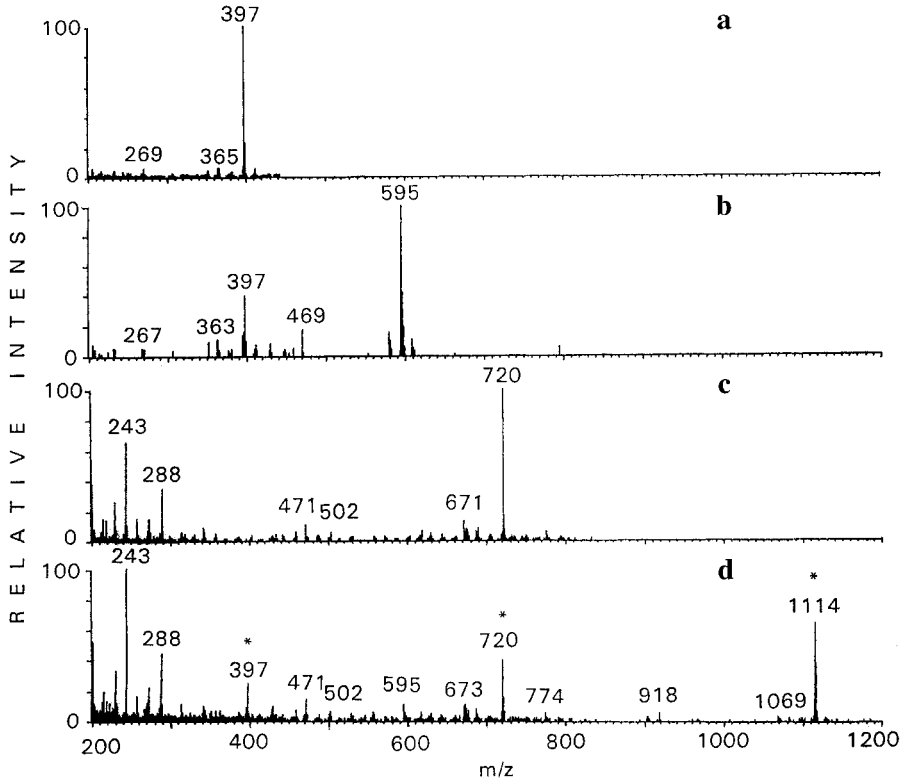

FIGURE 1: Positive fast atom bombardment ionization mass spectra of the linear peptide starting compounds and their reaction products. Note the dominant molecular ion (MH^+) present in each spectrum: (a) KCF (MH^+ at *m/z* 397); (b) activated (DNPT-)KCF (*m/z* 595); (c) CDVDIR (*m/z* 720); (d) disulfide-linked KCF-CDVDIR (*m/z* 1114). The intense fragments *m/z* 397 and 720 (molecular ions of the component peptides) are marked by * as diagnostic ions for this cystine-peptide.

followed by monitoring the development of the yellow color ($\epsilon_{408} = 12\,700\ M^{-1}cm^{-1}$).

The reactions were also followed by mass spectrometry using the conventional ("static") probe with the FAB technique (Figure 1). Figure 1a presents KCF before activation with the dominant molecular ion being *m/z* 397 (MH^+). The activated compound DNPT-KCF is presented in Figure 1b. Beside the dominant *m/z* 595 (MH^+), the molecular ion of the original non-derivatized KCF also appears in meaningful abundance. This ion is an MS artifact: the fast atom bombarded matrix material reduced part of the disulfide peptide. Reducing reactions, such as the opening of disulfide bonds in peptides (Visentini et al., 1989), dehalogenation of drugs (Edom et al., 1991), or demethylation of dyes (Gale et al., 1986) have often been discussed in the FAB-MS literature [e.g., Aubagnac et al. (1995) and references therein].

The third spectrum (Figure 1c) presents the "second" peptide CDVDIR with a dominant molecular ion at *m/z* 720, and the bottom panel (Figure 1d) presents the disulfide-coupled product KCF-CDVDIR. Beside the intense molecular ion of *m/z* 1114, the molecular ions of both reduced components *m/z* 720 (CDVDIR + H^+) and 397 (KCF + H^+) are exhibited. The three molecular ion peaks are important diagnostic tools in locating this cystine-peptide in the tryptic digest.

Additional helpful diagnostic data are the HPLC retention times. The chromatogram of the reaction product is shown in Figure 2a. The mass spectra from the fractions collected from the reverse phase HPLC were generated by CF-FAB mass spectrometry. The first peak with elution time of 27 min is the desired compound KCF-CDVDIR, as the corresponding mass spectrum indicates (Figure 2b; MH^+ at *m/z* 1114). The mass spectrum for the second peak (elution time, 37 min; Figure 2c) corresponds to activated DNPT-CDVDIR, (MH^+ at *m/z* 918). The third peak (elution time, 38 min) is the activated peptide DNPT-KCF (spectrum not shown here; it is identical to the one in Figure 1b). The mechanism that generated the second eluting compound is disulfide bond exchange or "scrambling" between the product KCF-CDVDIR and the excess reagent (third peak), a reaction which usually takes place in alkaline medium (Fontana et al., 1968a). It is important for the stability of the product that the pH of the solution be lowered to acidic values (pH 2−5) as soon as the coupling reaction is complete (1−5 min). The results of the disulfide bond exchange are seen also in the mass spectrum from the first HPLC peak (Figure 2) by the appearance of the symmetric cystine compounds' molecular ion *m/z* 791 (2KCF − 2H + H^+). Since both molecular ions *m/z* 1114 and 791 appear in the same fraction, the two peptide-compounds KCF-CDVDIR and KCF-KCF apparently have nearly identical retention times. We note that the KCF-CDVDIR spectrum prepared immediately after the coupling reaction (Figure 1d) does not exhibit this impurity.

Identifying KCF-CDVDIR in the Tryptic Digest of ISC β-Actin. Rechromatography of the KCF-CDVDIR peak fraction results in a single peak on the succeeding reverse phase HPLC run (Figure 3a). The retention time of 27 min provides the window used to select promising fractions from the ISC β-actin tryptic peptide map (Figure 3b). From the HPLC separation of the tryptic digest obtained from 250 ng (~6 nmol) of ISC actin, equivalent fractions were investigated by continuous-flow FAB-MS. All three diagnostic ion peaks, *m/z* 1114, 720, and 397, were found in the spectrum presented in Figure 3c. Although the ions of other peptides dominate the spectrum (e.g., MH^+ of T_{31}: ^{316}EITALAP-

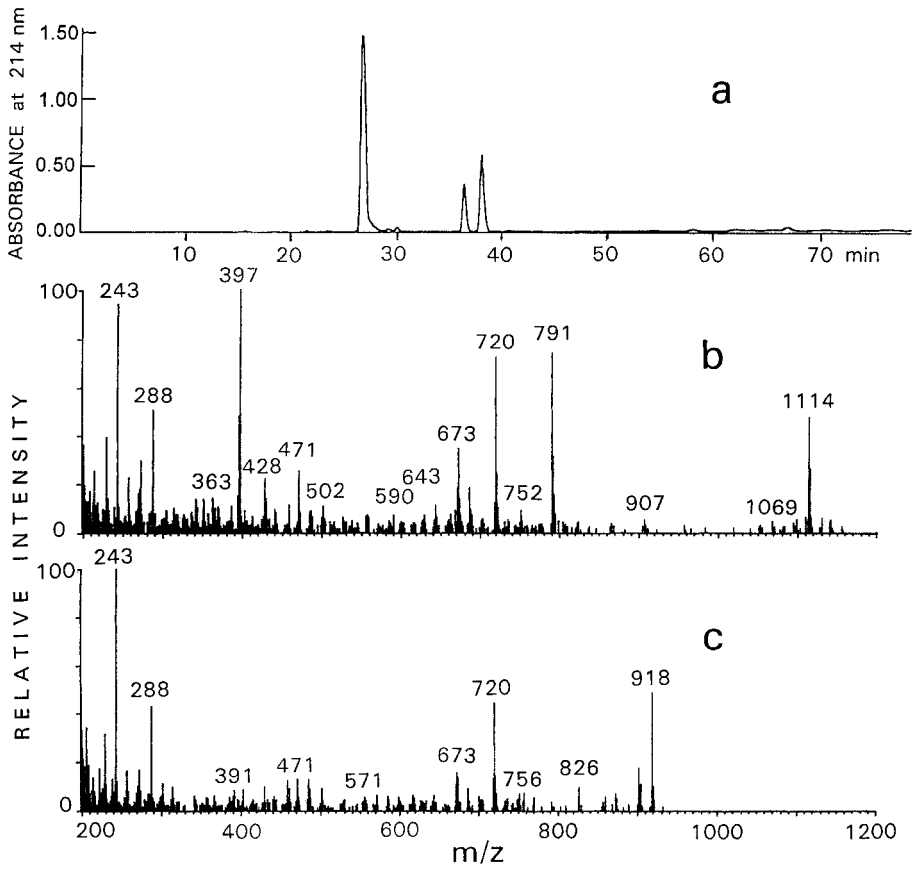

FIGURE 2: (a) HPLC trace of the raw products of the coupling reaction between activated KCF and CDVDIR. (b) Mass spectrum of the fast eluting compound, KCF-CDVDIR. (c) Mass spectrum of the second eluting compound, DNPT-CDVDIR. The third eluting compound is activated KCF, *i.e.*, excess reagent. The sample was stored at pH 8.0, 4 °C, for two weeks before chromatography.

STMK[326], *m*/*z* 1162), the diagnostic ions are easily distinguishable.

The MSMS product ion spectrum generated from the ISC β-actin tryptic molecular ion *m*/*z* 1114 (Figure 3d) has convincing similarity to the MSMS spectrum prepared from the synthetic KCF-CDVDIR (Figure 3e). The product ions present in both spectra, with definite structural meaning, are *m*/*z* 288 (Y''_2), 502 (Y''_4), and 1096 (B_6), if KCF-C is considered as one residue in the modified hexapeptide C*DVDIR (where C* = KCF-C). *m*/*z* 686 which appears in both spectra, was probably generated from *m*/*z* 720 (CDVDIR + H^+) by the loss of the H_2S side-chain. The ion *m*/*z* 752 in the spectrum of the model compound (Figure 3e) is probably holding both sulfur atoms from the parent molecular ion −S−S bridge. We regard the similarity of the two spectra as clear evidence of the identity of the two compounds they represent. That is, the β-actin from ISC RBC membranes does contain an S−S bridge between cysteine[284] and cysteine[373]. As we showed previously (Shartava et al., 1995), these cysteines are freely accessible in normal RBC actin for labeling reagents but are inaccessible in ISC actin. The current results are the first direct demonstration of a disulfide bridge between cysteine[284] and cysteine[373] in ISC β-actin. FAB-MS experiments with the corresponding HPLC fractions from a tryptic digest of control RBC β-actin indicated only minor quantities (<30% of that found in ISC β-actin) of the KCF-CDVDIR peptide based on the intensities of the diagnostic *m*/*z* 397, 720, and 1114 relative to *m*/*z* 1162. It appears that the formation of the cystine peptide is the result of an oxidative process which is well controlled by the healthy RBC but which is allowed to proceed to completion in ISCs containing diminished levels of reduced glutathione (Hebbel, 1990, 1991).

DISCUSSION

We demonstrate in the current work that a disulfide bridge does indeed exist between cysteine[284] and cysteine[373] of ISC β-actin. This result is consistent with our previous computer modeling of ISC β-actin (Shartava et al., 1995) based on the atomic structural model from X-ray crystallography of the bovine β-actin/profilin complex (Schutt et al., 1993). Our computer modeling suggested that the carboxy-terminal portion of actin (residues 372−374) undergoes a conformational change when its chaperon protein dissociates, which orients the C^{373} side chain toward the solvent and predisposes both C^{373} and C^{284} residues to disulfide bond formation. The fact that our previous measurement of available thiols in ISC β-actin gave a value of 0.2 mol/mol of actin, versus 2 mol of thiol/mol of actin in the control, suggested that at least 90% of the ISC β-actin would contain the disulfide bridge (Shartava et al., 1995). The current studies supply the first definitive proof by the identification of the KCF-CDVDIR cystine-linked peptide in the tryptic digest of ISC β-actin. Mass spectroscopy methods have previously been used to identify and localize disulfide bridges in insulin (Morris & Pucci, 1983), conotoxin G1, oxytocin, urotensin II, vasopressin (Yazdanparast et al., 1986), neurophysin (Burman et al., 1988), relaxin (Canova-Davis et al., 1991), thioltransferase (Papov et al., 1994), IL10 (Pannel et al., 1995), and synthetic peptides (Nemirovsky & Gross, 1995).

The discovery of the disulfide-linked KCF-CDVDIR peptide was based on our ability to prepare the synthetic

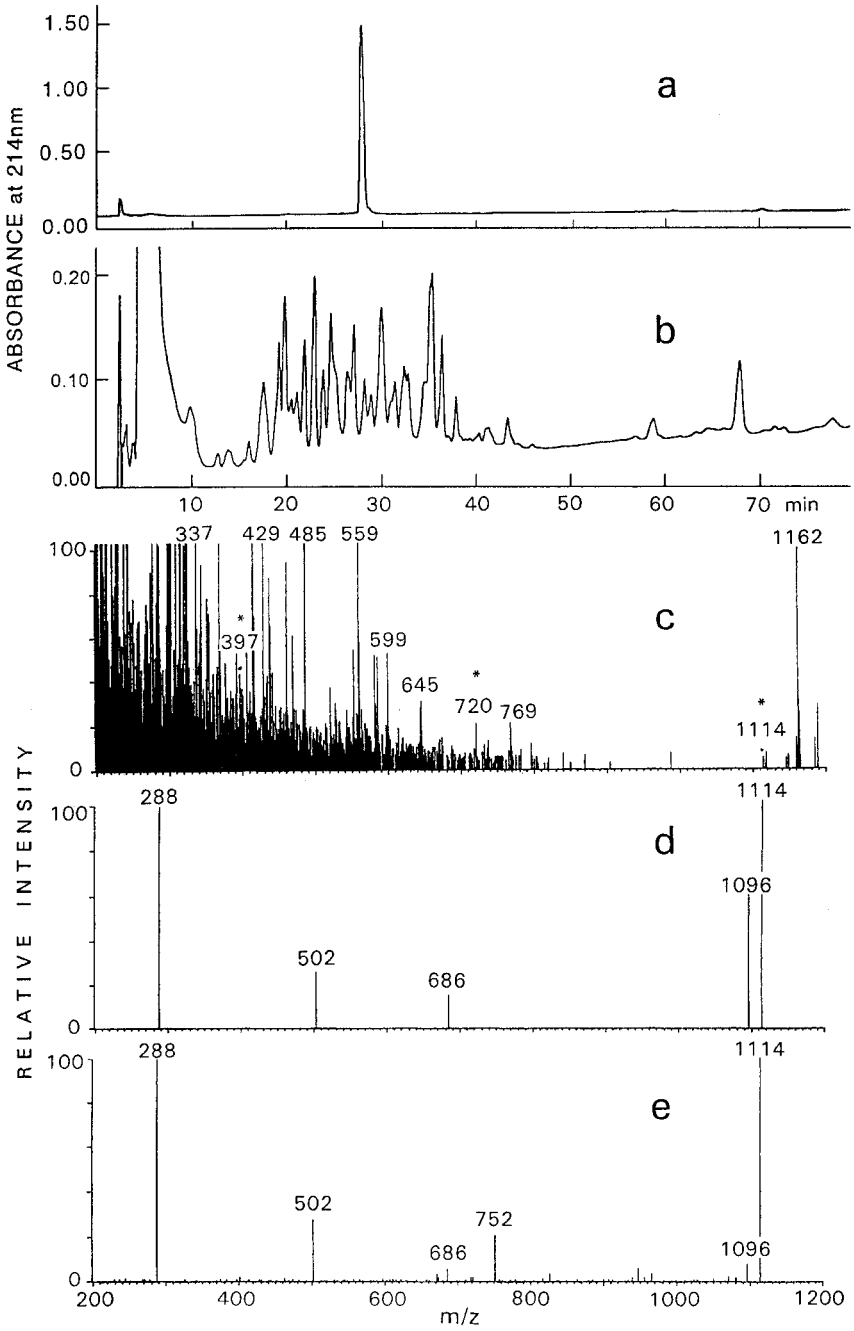

FIGURE 3: (a) HPLC trace of synthetic KCF-CDVDIR. (b) ISC β-Actin peptide map: HPLC trace of a tryptic digest from 250 ng of protein. (c) FAB mass spectrum of fraction 27 from the tryptic digest, the diagnostic ions for KCF-CDVDIR are marked by *. (d) MSMS product ion spectrum of *m*/*z* 1114 generated from the tryptic digest fraction 27. (e) MSMS product ion spectrum of *m*/*z* 1114 generated from synthetic KCF-CDVDIR.

peptide. Several facts about the chemistry of aromatic sulfenyl halides are worth noting and should be helpful to those working to prepare cystine-linked synthetic peptides. (1) Nitro- and dinitrosulfenyl chloride react not only with thiol groups but also with indole moieties (Scoffone et al., 1968). Thus not only cysteine but also tryptophan can be modified. This latter reaction quantitatively (and irreversibly) transforms the indole moieties into 2-(2′,4′-dinitrophenyl)thioindole derivatives. Azobenzenesulfenyl bromide, however, reacts solely with the sulfhydryl groups (Fontana et al., 1968b) but is far more expensive that the dinitrosulfenyl chloride. Since our peptides did not contain tryptophan, the specificity of DNPS-Cl was adequate for our experiments. (2) The aromatic sulfenyl halides are fairly hydrolysis resistant compounds, and therefore the thiol activation step does not require absolute conditions. In fact, the reaction may be carried out in 50% aqueous acetic acid solution (Scoffone et al., 1968). (3) According to the Fontana method (1968a,b), the reaction mixture is poured in ether at the end of the activation reaction and the precipate is filtered. Because of the small amounts of peptide with which we started the reaction (4 mg of KCF), we replaced the ether with a less volatile mixture of tetrahydrofurane and toluene (1:10) and performed the filtration by centrifugation. These modifications allowed us to carry out the reaction in one single centrifuge glass at ambient temperature, with an excellent overall yield (80%).

Experiments are currently underway in our laboratory to determine whether actin's role in the slow dissociation of the ISC membrane skeleton is due to an altered actin−actin

and/or actin−spectrin interaction. In addition, we have recently demonstrated that a reducing agent, DTT, has the ability to increase the rate constant for dissociation of the ISC membrane skeleton to control values, block the formation of ISCs *in vitro*, and convert ISCs formed *in vivo* to biconcave cells (Goodman et al., 1995). Therefore, the disulfide bridge between cysteine^{284} and cysteine^{373} in ISC β-actin appears to be critical in ISC formation and could be the target of future therapies designed to block ISC formation in sickle cell subjects.

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