Physical Properties of a Single-Motif Erythrocyte Spectrin Peptide: A Highly Stable Independently Folding Unit†

Tara M. DeSilva,‡,§ Sandra L. Harper,‡ Leszek Kotula,‡,|| Preston Hensley,⊥ Peter J. Curtis,‡ Laszlo Otvos, Jr.,‡ and David W. Speicher*,‡

The Wistar Institute, Philadelphia, Pennsylvania 19104, and SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

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ABSTRACT: Spectrin is a long flexible rod-like actin cross-linking protein mostly comprised of many tandem homologous 106-residue motifs. In this study, the conformational stability and physical properties of a single homologous motif peptide, α1, were evaluated and compared to intact spectrin monomers and αβ heterodimers. It is interesting that while spectrin dimers elongate by about 3-fold in low ionic strength buffers relative to their size in physiological buffers, the single-motif peptide does not show significant changes in secondary structure in 10 mM phosphate buffer compared with isotonic buffer. This single-motif peptide is monomeric in physiological buffer as demonstrated by equilibrium sedimentation studies, and its hydrodynamic radius determined by gel filtration and dynamic light scattering of about 2.2 nm is consistent with an elongated rod-like shape. Unfolding of the single-motif peptide in urea solutions was similar to unfolding of intact heterodimers. Differential scanning calorimetry analyses showed that this single motif undergoes a reversible two-state transition with a T_m of 53 °C and an enthalpy of 65 kcal/mol in physiological buffer. Thermal stability was unaffected by ionic strength changes, but was decreased below physiological pH. These data show that this 13 kDa spectrin motif is a monomeric, highly stable, triple-helical, independently folding protein building block with physical characteristics that define many of the structural properties of the 526 kDa spectrin heterodimer. In contrast, interactions between adjacent motifs are probably responsible for spectrin's molecular flexibility and elasticity.

Spectrin is the predominant protein in the erythrocyte membrane skeleton where it plays an integral role in maintaining the erythrocyte's biconcave shape, membrane flexibility, and membrane elasticity. It is comprised of two large subunits, α (280 kDa)[1] and β (246 kDa), which associate laterally in an antiparallel orientation to form heterodimers. This side-to-side association is dependent upon nucleation site regions located near the C-terminal of the α subunit and near the N-terminal of the β subunit (Speicher *et al.*, 1992; Ursitti *et al*., 1996). Two spectrin heterodimers further associate head-to-head to form tetramers, the predominant form of spectrin on the erythrocyte membrane. Perturbations in this critical interaction are responsible for many hemolytic anemias [see Lux and Palek (1995) for a recent review].

Purified spectrin dimers are flexible rod-like molecules with a contour length of about 100 nm as indicated by electron microscopy (Shotton *et al*., 1979), which is similar to the length observed for spectrin in Triton-extracted red cell membrane skeletons under low ionic strength conditions (Byers & Branton, 1985; Shen *et al.*, 1986; Liu *et al.*, 1987). Several studies have shown that the shape and stability of erythrocyte membrane Triton skeletons are affected by varying ionic strength, pH, temperature, concentration, and chaotropic agents, presumably due primarily to changes in spectrin's molecular shape and intermolecular interactions (Johnson *et al*., 1980; Lange *et al*., 1982; Vertessy & Steck, 1989; Svoboda *et al*., 1992). The isolated spectrin molecule is also highly dynamic (Mikkelsen & Elgsaeter, 1978; Mikkelsen *et al*., 1984; Fung *et al*., 1986; Clague *et al*., 1990; Budzynski *et al*., 1992), which is consistent with spectrin's dominant role in contributing to the flexibility and integrity of the erythrocyte membrane. Apparently, spectrin's flexibility and ability to change its molecular shape must be conferred in some manner by the many homologous motifs of approximately 106 amino acids that comprise most of the dimer's mass as indicated by amino acid and cDNA sequencing (Speicher & Marchesi, 1984; Sahr *et al*., 1990; Winkelmann *et al*., 1990).

A triple-helix model was initially proposed for the homologous spectrin motifs (Speicher & Marchesi, 1984), which was consistent with the high α-helical content observed for erythrocyte spectrin dimers using circular dichroism (Calvert *et al*., 1980). Subsequently, X-ray crystallography of the 14th homologous motif of *Drosophila* α spectrin (Yan *et al*., 1993) confirmed that spectrin motifs

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* To whom correspondence should be addressed at The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104. Phone: 215-898-3972. Fax: 215-898-0664.

‡ The Wistar Institute.

§ Present address: Department of Chemistry, University of Pennsylvania, 231 S. 34th St., Philadelphia, PA 19104.

|| Present address: Laboratory of Molecular Neurobiology, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY 10314.

⊥ SmithKline Beecham Pharmaceuticals.

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[1] Abbreviations: Da, dalton(s); PBS, phosphate-buffered saline; LB, luria broth; HPLC, high-performance liquid chromatography; CD, circular dichroism; DSC, differential scanning calorimetry; PMSF, phenylmethanesulfonyl fluoride; GST, glutathione *S*-transferase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MALDI, matrix-assisted laser desorption/ionization.

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have a triple-helical conformation and provided the first high-resolution structure of a spectrin motif. However, this single-motif *Drosophilia* peptide crystallized as an artifactual homodimer where the C helix of one peptide paired in an antiparallel orientation with a second peptide to form an antiparallel homodimer. Ralston *et al*. (1996) subsequently demonstrated that this α14 single-motif peptide also exists as a homodimer in solution under some conditions.

A critical feature in understanding the structure and function of a single spectrin motif unit is determining the boundaries for complete stable folding units. The boundaries for complete folding units of spectrin homologous motifs were initially determined for *Drosophilia* spectrin using recombinant proteins (Winograd *et al*., 1991). This analysis determined that the starting point for motifs was offset by 26−30 amino acids from the original starting point of alignments that had been proposed based on sequence homology. However, Lusitani *et al*. (1994) subsequently expressed the first homologous motif of human erythrocyte spectrin (residues 49−155), using the phasing determined by Winograd and co-workers, and found that this motif was not stable and was rapidly degraded by proteases. In contrast, a very similar peptide from the first motif (α1) of human erythrocyte α spectrin, residues 50−158, was shown to be native using circular dichroism and was protease resistant (Kotula *et al*., 1993).

In the current study, we characterized the conformational stability of the human spectrin α1 single-motif recombinant peptide (residues 50−158) using multiple physical methods and compared its properties to intact spectrin monomers and dimers. These experiments show that the triple-helical α1 motif is a highly stable independently folding monomeric species that defines many of spectrin's structural properties.

MATERIALS AND METHODS

Purification of Spectrin Dimers. Crude spectrin was isolated from donor blood within 24 h of collection as previously described (Speicher *et al*., 1992). Crude spectrin was concentrated by precipitation in 50% ammonium sulfate, resuspended in 20−30 mL of isotonic KCl buffer [10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM 2-mercaptoethanol, and 30 μM phenylmethanesulfonyl fluoride (PMSF), pH 7.4], and dialyzed in the same buffer. Spectrin dimers were purified by chromatography of this sample on a Sepharose CL-4B column (5 × 90 cm).

Expression and Purification of the α1 Recombinant Peptide. The construction of a pGEX-2T plasmid expressing a GST-α1 fusion protein (α spectrin residues 50−158) was previously described (Kotula *et al*., 1993). Transfected DH5α cells were grown overnight at 37 °C, and then diluted 1:20 in LB medium containing 50 μg/mL ampicillin. After cells reached an optical density of 0.5−0.8 at 550 nm, they were induced with 1 mM 1-thio-β-D-galactopyranoside (IPTG) for 3 h, collected by centrifugation at 3000*g*, and stored at −80 °C. Purification of fusion proteins was carried out essentially as described by Kennedy *et al*. (1991) with modifications. Briefly, cell pellets from 600 mL cultures were resuspended in 15 mL of lysis buffer (50 mM Tris, 50 mM NaCl, 5 mM EDTA, 1 mM DFP, 0.15 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1% Triton X-100, pH 8.0). Cells were lysed by sonication; the supernatant was collected by centrifugation at 20000*g* and loaded directly onto a reduced glutathione−Sepharose 4B column (Pharmacia). The recombinant peptide fusion protein was eluted with 50 mM Tris, 10 mM reduced glutathione, pH 8.0. The α1 peptide was cleaved from the fusion protein in the elution buffer with bovine thrombin (Sigma), 5 units/mg, for 4 h at 37 °C. Cleaved peptides were purified from the glutathione moiety by rechromatography on the glutathione−Sepharose column. The unbound peak was concentrated using a 10K Centriprep concentrator (Amicon) and further purified by HPLC preparative gel filtration using two columns in series (G3000SW + G2000SW, 21.5 × 600 mm, TosoHaas) in phosphate-buffered saline (PBS) (130 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 0.15 mM PMSF, and 0.05% sodium azide, pH 7.4).

Analytical HPLC Assay. Analytical evaluation of α1 purity and the Stokes radius was determined using two silica-based HPLC columns in series (TSK G3000 SW_{XL}, G2000 SW_{XL}, 7.8 × 300 mm). The columns and injector were maintained at 4 °C, and a flow rate of either 0.4 or 0.8 mL/min was used with PBS.

Polyacrylamide Gel Electrophoresis. Purification of the α1 peptide was monitored using 15% Tricine gels (Schagger & von Jagow, 1987) stained with Coomassie Blue.

Circular Dichroism (CD) Measurements. Protein concentrations were determined by quantitative amino acid analysis shortly before CD measurements. CD spectra were obtained on a Jasco J720 instrument at room temperature in a 0.2 mm path length cell. Curve smoothing was performed using Jasco software (1.10c).

Differential Scanning Calorimetry (DSC). DSC measurements were performed using a MicroCal MCS calorimeter. Protein solutions were dialyzed against the appropriate buffer and were degassed for approximately 10 min prior to loading the sample cell. An aliquot of the dialysate was used in the reference cell. Peptide concentrations varied between 0.74 and 1.1 mg/mL. All scans were from 10 to 90 °C at a scan rate of 90 °C/h. ORIGIN software (MicroCal, Inc.) was used for data analysis, which involved subtracting a buffer base line from the raw data and then defining a base line using a progress curve fitted to the end points of the transition. The constructed base line was then subtracted, and the data were curve-fitted using standard models to determine T_m and the heat capacity associated with the thermal unfolding.

RESULTS

Purification and Characterization of the α1 Spectrin Motif Recombinant Peptide. The location of the first complete motif of α spectrin, α1 (residues 50−158), and its relationship to spectrin heterodimers are shown in Figure 1. The α1 peptide was purified and cleaved from its fusion protein in good yield. Approximately 50 mg of fusion protein was typically obtained per liter of bacterial culture, which yielded about 8 mg of purified α1 peptide (about 50% of the theoretical yield based on masses) after thrombin cleavage and repurification (Figure 2).

Evaluation of spectrin by equilibrium sedimentation in a Beckman XL-A analytical ultracentrifuge showed that the α50−158 peptide was a monomeric species at 25 °C in PBS (Figure 3). The fitted value for the molecular mass in this experiment was 13 246 Da, which compared favorably with the predicted mass of 13 177 Da calculated from the sequence. These data demonstrated that at least up to a

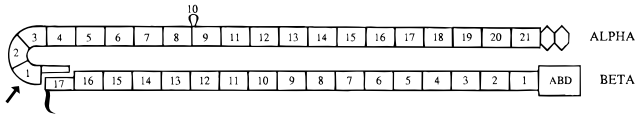

FIGURE 1: Motif model of a spectrin heterodimer. A schematic model of the motifs that comprise an antiparallel spectrin dimer is shown. The α1 motif is indicated by an arrow, and is the first complete homologous motif of α spectrin (amino acids 50−158). It is preceded by an N-terminal region, residues 1−49 (unlabeled narrow rectangle), which is probably a partial homologous motif consisting primarily of a single helix that binds in a head-to-head fashion with the two helices of the *β* spectrin motif 17 to form the tetramer binding site (Tse *et al.*, 1990; Speicher *et al.*, 1993; Kennedy *et al.*, 1994). In addition to this partial motif, the α subunit consists of 20 homologous motifs (numbered rectangles), an SH3 type motif (motif 10) that is actually located in the connecting loop between helices B and C of motif 9, and a carboxyl-terminal region, which is predominantly two EF-hand motifs (diamonds). The *β* subunit, which pairs with the α subunit in an antiparallel orientation, consists of an actin binding domain (ABD), 16 complete homologous motifs, a partial 2-helix 17th motif, and a phosphorylated C-terminal domain (squiggle).

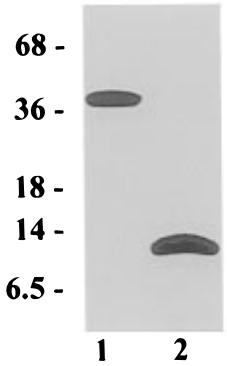

FIGURE 2: Characterization of the recombinant α1 single-motif peptide. Proteins (2 *μ*g/lane) were separated on a 15% Tricine gel. Lane 1, the recombinant GST−α1 fusion protein after purification on a glutathione column. Lane 2, the α1 peptide after cleavage from the GST moiety, rechromatography on the glutathione−Sepharose column, and further purified by gel filtration.

concentration of 9×10^{-5} M, there was no evidence of an artifactual homodimeric species. Analysis of the α1 peptide mass using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry also confirmed the expected mass of the recombinant peptide (data not shown).

The purified α1 peptide migrated as a single discrete band on a gel and was stable for at least several months when stored at 0 °C in physiological buffer. Analytical HPLC gel filtration analysis showed that the α1 peptide migrated as a single sharp peak with no apparent formation of dimers or oligomers, or aggregation (data not shown). A Stokes radius of 2.3 nm was determined by calibrating these gel filtration columns with standard proteins. In addition, dynamic light scattering measurements of α1 at 5 mg/mL in PBS using a DynaPro-801 instrument (Protein Solutions, Inc.) showed that the peptide was monodisperse with a Stokes radius of 2.0 nm, which is in good agreement with the value determined by gel filtration. Since the peptide is a monomer as shown by equilibrium sedimentation, the Stokes radii values determined by gel filtration and dynamic light scattering are consistent with an asymmetric rod-like shape.

CD Measurements of the α1 Motif and Intact Spectrin Dimers at Different pHs. CD spectra of the α1 recombinant peptide and spectrin dimers were obtained at different pHs (Figure 4). The total helix content of the single-motif α1

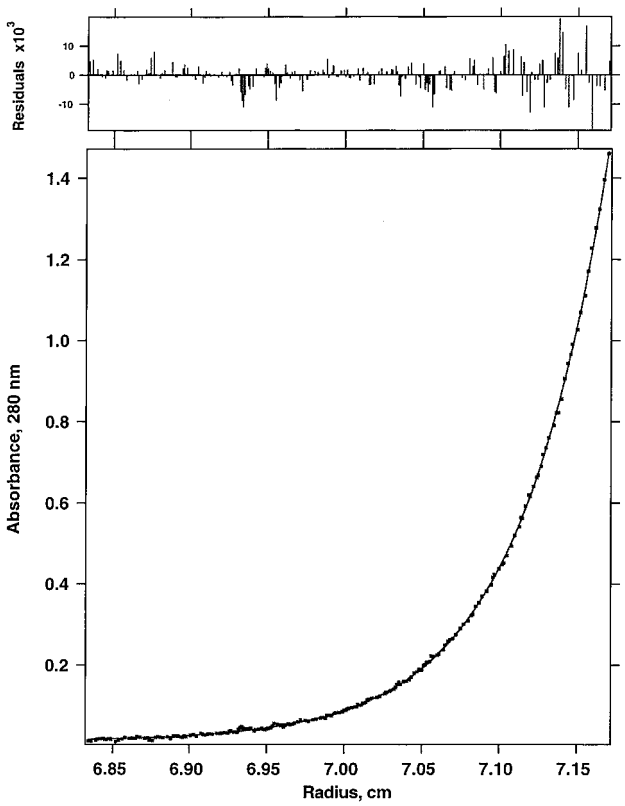

FIGURE 3: Equilibrium sedimentation of the recombinant α1 peptide. The α1 peptide at an initial concentration of 0.43 mg/mL in PBS at pH 7.4 was analyzed by equilibrium analytical ultracentrifugation. Samples were centrifuged at 40 000 rpm at 25 °C for 34 h in a Beckman XL-A analytical ultracentrifuge. Data were analyzed in terms of a single sedimenting species as previously described (Chan *et al.*, 1996; Brooks *et al.*, 1994), using a value of 0.734 mL/g for the partial specific volume, computed by the method of Cohn and Edsall as described by Laue *et al.* (1992). The fitted value for the molecular mass was 13 246 ± 30 Da.

peptide as well as intact spectrin dimers was unaffected by pH changes ranging from 5.9 to 9.4. However, the α1 peptide, but not intact spectrin dimers, showed a variable positive $\pi\pi^*$ band (far-UV region) at different pHs. Both the α1 single-motif peptide and the intact dimer had an estimated α helical content of about 75−80% as estimated by the mean residue ellipticity at 208 nm (Greenfield & Fasman, 1969). Below pH 5.9, both the α1 peptide and spectrin dimers precipitated, presumably because the isoelectric points of both samples are approximately pH 5.2. CD measurements at different pHs of intact α monomers and an α subunit fragment containing the first six homologous motifs were similar to the spectra observed for spectrin dimers (data not shown).

Influence of Ionic Strength on Secondary Structure. As described above, spectrin molecules assume a dramatically extended molecular shape in low ionic strength buffers compared with physiological buffers. These changes in molecular size could occur either via reorientations between adjacent homologous motifs or through changes within the triple-helical motif itself; however, no changes in dimer secondary structure as a function of ionic strength were previously observed (Calvert *et al.*, 1980; LaBrake *et al.*, 1993). To determine whether ionic strength changes could affect the conformation of a single-motif spectrin peptide, CD measurements of the α1 peptide were performed at physiological and low ionic strength conditions (10 mM

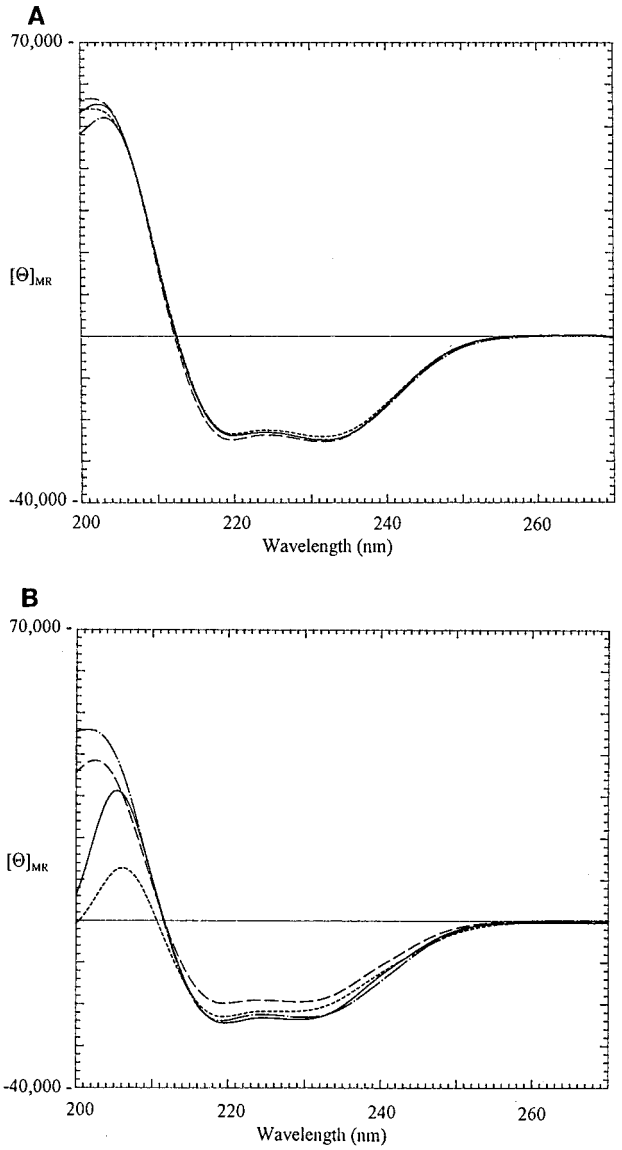

FIGURE 4: Circular dichroism of the α1 single-motif peptide and spectrin dimers at different pHs. Samples of α1 peptide and full-length dimers in PBS at the indicated pHs were analyzed. Concentrations of samples were determined using quantitative amino acid analysis prior to each CD measurement. Mean residue ellipticity, $[\theta]_{MR}$, is expressed in degrees centimeter squared per decimole. Spectrin heterodimers (A) and the α1 peptide (B) were analyzed under the following conditions: (− −) pH 8.4; (—) pH 7.4; (- - -) pH 6.4; (− · −) pH 5.9. Samples were also analyzed at pH 9.4 (data not shown); these spectra were similar to those shown for pH 8.4.

sodium phosphate) where the intact dimer elongates by about 3-fold. As shown in Figure 5, the helical contents of both the α1 motif and intact spectrin dimers were unaffected by this difference in ionic strength.

Urea Denaturation of the α1 Peptide. CD was used to evaluate unfolding of the α1 single-motif peptide by urea. Varying concentrations of urea were added to protein samples in phosphate-buffered saline at pH 7.4, and samples were then incubated overnight at 0 °C prior to CD analysis. Samples were warmed to room temperature immediately before loading the CD cuvette, and the mean residue ellipticity at 222 nm was determined (Figure 6). This single-motif peptide showed similar denaturation properties to the intact dimer, which was consistent with independent unfolding of individual motifs in the intact molecule. The α1

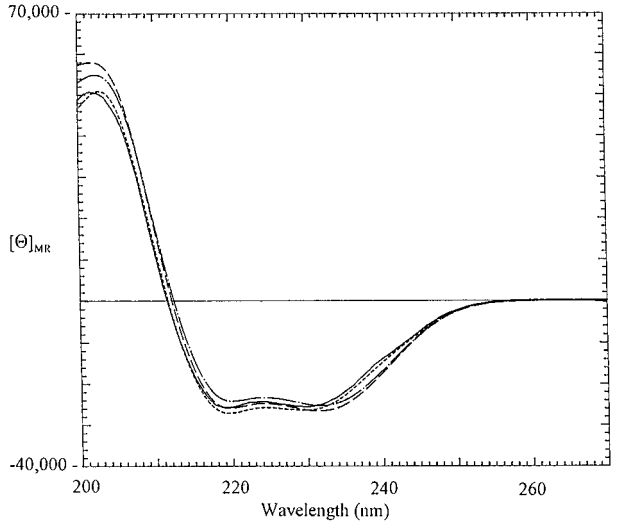

FIGURE 5: Ionic strength effects on the secondary structure of the α1 single-motif peptide and spectrin dimers. The proteins were dialyzed into either 10 mM sodium phosphate, pH 7.4, or PBS, pH 7.4. Protein concentrations were determined by amino acid analysis prior to CD measurements. Mean residue ellipticity, $[\theta]_{MR}$, is expressed in degrees centimeter squared per decimole. (—) α1 peptide in 10 mM sodium phosphate; (- - -) α1 peptide in PBS; (− −) spectrin dimer in 10 mM sodium phosphate; (− · −) spectrin dimer in PBS.

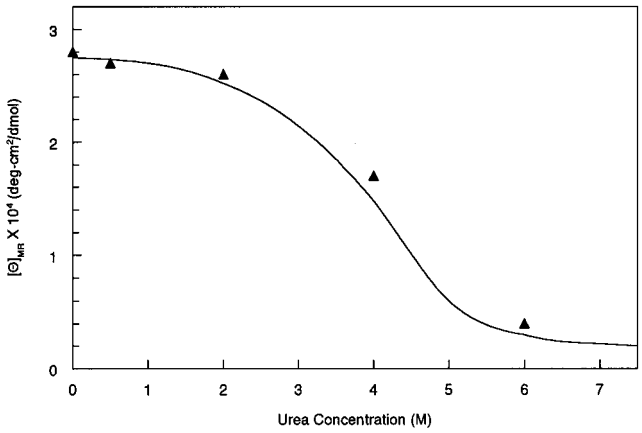

FIGURE 6: Unfolding of the α1 peptide in urea solutions. Molar residue ellipticities at 222 nm for the α1 peptide (▲) at various urea concentrations are shown superimposed on a curve (—) for spectrin dimer unfolding in urea taken from Calvert *et al.* (1980).

peptide could refold readily after denaturation in 6 M urea since the Stokes radius observed after urea was removed by dialysis was identical to untreated controls (data not shown).

Thermal Denaturation of the α1 Peptide. DSC experiments of the single-motif α1 peptide in PBS, pH 7.4, at about 1 mg/mL showed a single reversible two-state transition. The peptide was surprisingly highly stable with a $T_m = 53.4$ °C and an estimated enthalpy change of about 65 kcal/mol. Several multiple-motif recombinant peptides as well as α monomers showed more complex thermal denaturation curves that were not reversible (data not shown).

The effects of varying pH on the thermal unfolding of the α1 recombinant at physiological ionic strength are shown in Figure 7. Thermal denaturation was minimally altered at pH 8.4 compared with physiological pH. In contrast, the thermal transition broadened substantially, and the transition midpoint was shifted to about 41 °C at pH 6.4. Since the helical content of α1 was unaffected by this pH change, the

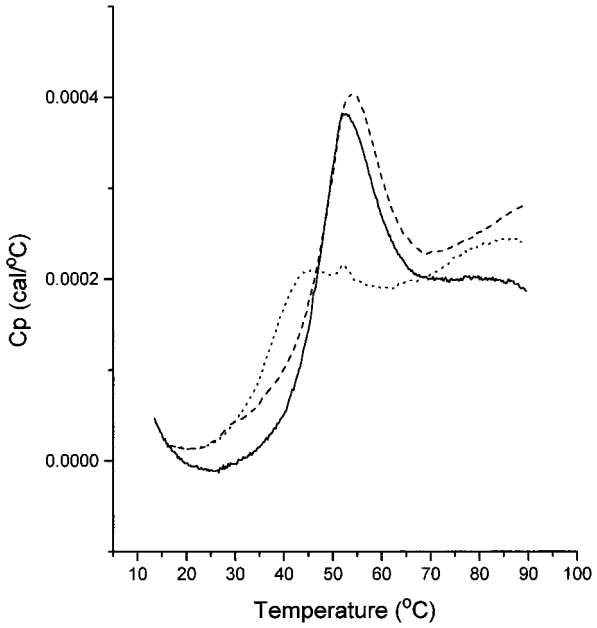

FIGURE 7: Effects of pH on thermal unfolding of the single-motif α1 peptide. Samples were dialyzed into PBS at the desired pH and analyzed by DSC at 0.8 mg/mL using a scan rate of 90 °C/h. (- - -) pH 8.4; (—) pH 7.4; (•••) pH 6.4.

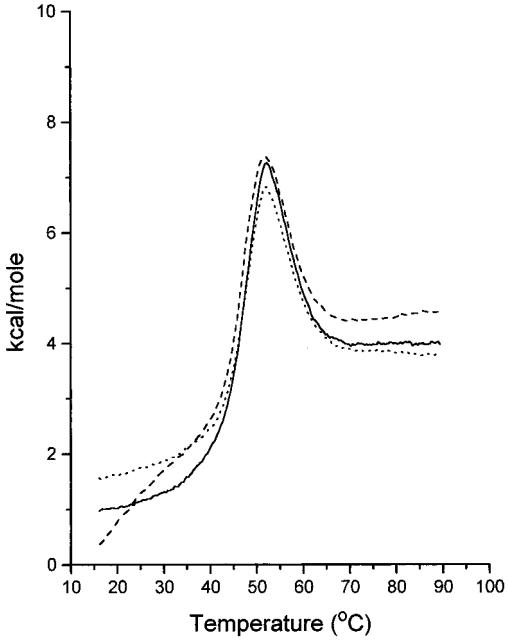

FIGURE 8: Effects of ionic strength on thermal unfolding of the α1 recombinant peptide. Samples were dialyzed into a 5 mM sodium phosphate buffer at pH 7.4 containing the indicated NaCl concentration and were analyzed by DSC at 0.95 mg/mL using a scan rate of 90 °C/h. (—) 0 mM NaCl; (•••) 150 mM NaCl; (- - -) 500 mM NaCl.

decreased thermal stability observed at pH 6.4 compared with pH 7.4 suggests changes in tertiary packing of the three helices in the triple-helical motif at the lower pH. Thermal denaturation of the α1 peptide remained reversible at all pHs shown, and no precipitation was observed in these experiments.

Although ionic strength changes did not affect the secondary structure of the single-motif α1 peptide as determined by CD, one possible mechanism for producing the observed elongation of spectrin monomers and dimers in low ionic strength buffers would involve reorientation of the three helices in the triple-helical bundle. To further explore this possibility, the effects of ionic strength on thermal unfolding of the α1 peptide at pH 7.4 were evaluated (Figure 8). These experiments showed that large ionic strength differences (0−500 mM NaCl) did not change the thermal stability of this single-motif peptide, which exhibited a T_m of 53 °C for all conditions tested. These experiments indicate that ionic strength induced changes in interhelical pairing within a triple-helical motif probably do not occur and are not responsible for molecular shape changes associated with changes in ionic strength. The lack of an effect on thermal stability of ionic strength changes suggests that ionic interactions do not appreciably contribute to stabilization of the tertiary structure of this motif.

DISCUSSION

Spectrin is a 526 kDa αβ heterodimer where both chains are comprised primarily of many homologous tandem motifs that are presumed to confer most of spectrin's molecular properties, including: its ability to rapidly renature, its elongated shape, its flexibility, and its ability to change length in low ionic strength buffers and when under tensile stress. Hence, analysis of the physical properties of these 106-residue protein building blocks with masses of about 12−13 kDa should provide valuable insights into properties of the very large αβ spectrin dimer.

One of the striking features of spectrin structure that emerged from early studies of its physical properties was the relative ease and fidelity of renaturation of the very large spectrin monomers and dimers after unfolding with urea (Calvert *et al*., 1980; Yoshino & Marchesi, 1984). This facile renaturation was attributed to the independent refolding of multiple domains, which was a perceptive interpretation since these studies preceded the discovery that most of the mass of both spectrin subunits is comprised of many homologous 106-residue segments. The current study directly demonstrates that a single homologous segment can independently fold into a highly stable conformation with a number of physical properties that closely reflect the behavior of the much larger spectrin heterodimer.

As briefly discussed above, several previous studies examined single-motif or several-motif spectrin peptides, primarily using mild proteolysis to evaluate polypeptide chain folding and stability. Initially, Winograd *et al.* (1991) determined the motif boundaries (phasing) for stable recombinant *Drosophila* spectrin peptides. However, Lusitani *et al*. (1994) used this phasing to express the first homologous motif of human erythrocyte α-spectrin (residues 49−155) and found that it was not very stable to proteases. In contrast, the current study demonstrates that a very similar human erythrocyte α-spectrin peptide (residues 50−158) is a quite stable triple-helical motif. The important difference between these two latter studies appears to be the additional three amino acids on the C-terminal end of the peptide used in the current study.

An important nuance of the Winograd *et al.* (1991) study is that they routinely added an extra two residues to the ends of their peptides relative to their predicted motif boundary. Peptides that end precisely at the putative boundary may not fold properly, and the marked differences in stability between the α49−155 and α50−158 peptides suggest that a truncated C-terminal boundary in the motif may be particularly

destabilizing. These observations are consistent with an analogous evaluation of the slightly larger, but evolutionarily related, spectrin-like motifs of dystrophin by Kahana and Gratzer (1995), who showed that the precise length of the C helix dramatically affected the motif's stability. They concluded that the C-terminal length was more important than the precise residue at the C-terminal since they found that a wild-type C-terminal glutamine could be replaced with a methionine without affecting the conformation, but deletion of the C-terminal residue led to a large reduction in helical content.

We have also observed that extra residues on the C-terminal ends of some spectrin recombinant peptides do not adversely affect folding or functional properties of recombinant spectrin peptides (Ursitti *et al*., 1996). A general rule that appears to be emerging is that spectrin motif peptides that are too short, especially on their C-terminal end, may be greatly destabilized, while the presence of a few extra residues has no negative effect.

Another important physical feature of spectrin is its ability to extend its molecular length by about 3-fold in low ionic strength buffers and when under tensile stress. The submolecular basis of this elongation is not yet clearly defined; but based upon this study, molecular elongation in low ionic strength buffers apparently does not involve appreciable changes in either the secondary or the tertiary structure of the individual motifs since both the CD and DSC analyses of the α1 peptide were unaffected by ionic strength changes. This strongly suggests that spectrin's elongation property arises from changes in interactions at the interfaces between adjacent homologous motifs rather than within the motifs.

The secondary and tertiary structures of the α1 single-motif spectrin peptide appeared to be relatively unaffected by higher than physiological pHs. In contrast, thermal stability was much lower at pH 6.4 as reflected by the shift in the thermal denaturation midpoint from 53 °C to about 41 °C. Also, while the total helicity over a pH range from 5.9 to 9.4 remained constant, changes were observed in the positive $\pi\pi^*$ band at different pHs, and the greatest decrease in this band occurred at pH 6.4 (Figure 4B), which may indicate a change in helix lengths or possibly a loss of helix−helix interactions. Taken together, these two observations suggest that a substantial destabilizing change in tertiary structure occurs at this pH.

It is interesting that urea-induced denaturation of the single-motif α1 peptide monitored by CD closely resembles denaturation of intact spectrin heterodimers (Figure 6). The observed unfolding of this single-motif peptide over a relatively broad range of urea concentrations is somewhat surprising since thermal unfolding fits a single two-state transition. In this regard, it is interesting that the urea denaturation curve for the α1 spectrin peptide observed in this study is nearly identical to CD data obtained for urea denaturation of a protease-resistant, single-motif dystrophin peptide with the proper phasing (Kahana *et al.*, 1994). These comparable results for single-motif spectrin and dystrophin peptides as well as full-length spectrin dimers suggest that the conformational stability of most homologous spectrin-like motifs may be quite similar, and intermotif interactions appear to minimally contribute to the conformational stability of spectrin.

The thermal stability of the α1 peptide indicated by the DSC experiments with a T_m of 53 °C is actually surprisingly high. This single domain is somewhat more stable than intact spectrin heterodimers or isolated monomers, which denature at about 49 °C (Yoshino & Marchesi, 1984). The significance of this difference is not clear at present, and further DSC analyses of other single-motif and multiple-motif peptides are required to more clearly elucidate the role of individual motifs and intermotif interactions in thermal denaturation of spectrin.

In summary, the human erythrocyte spectrin α1 single-motif recombinant peptide has similar secondary structure to α monomers and α−β heterodimers over a range of ionic strengths and pHs, and it shows similar unfolding in the presence of urea. The single-motif peptide is monomeric in physiological buffer as demonstrated by equilibrium sedimentation. Its hydrodynamic radius determined by gel filtration and dynamic light scattering is about 2.2 nm, which is consistent with an asymmetric shape. Differential scanning calorimetry showed that this single motif is highly stable at physiological pH with a T_m of 53 °C that is unaffected by ionic strength changes. These data show that the triple-helical α1 motif is a highly stable independently folding monomeric species that largely determines spectrin's conformational stability and its ability to facilely refold. However, interactions between adjacent motifs are probably responsible for spectrin's molecular flexibility and ionic strength sensitive shape changes since this single motif has an elongated rod-like shape that is unaffected by changes in ionic strength.

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REFERENCES

Brooks, I., Watts, D. G., Soneson, K. K., & Hensley, P. (1994) *Methods Enzymol. 240*, 459−478.

Budzynski, D. M., Benight, A. S., LaBrake, C. C., & Fung, L. W.-M. (1992) *Biochemistry 31*, 3653−3660.

Byers, T. J., & Branton, D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6153−6157.

Calvert, R., Ungewickell, E., & Gratzer, W. (1980) *Eur. J. Biochem. 107*, 363−367.

Chan, W., Helms, L. R., Brooks, I., Lee, G., Ngola, S., McNulty, D., Maleeff, B., Hensley, P., & Wetzel, R. (1996) *Folding Des. 1*(2), 77−89.

Clague, M. J., Harrison, J. P., Morrison, I. E. G., Wyatt, K., & Cherry, R. J. (1990) *Biochemistry 29*, 3898−3904.

Fung, L. W.-M., Lu, H.-Z., Hjelm, R. P., Jr., & Johnson, M. E. (1986) *FEBS Lett. 197*, 234−238.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108−4116.

Johnson, R. M., Taylor, G., & Meyer, D. B. (1980) *J. Cell Biol. 86*, 371−376.

Kahana, E., & Gratzer, W. B. (1995) *Biochemistry 34*, 8110−8114.

Kahana, E., Marsh, P. J., Henry, A. J., Way, M., & Gratzer, W. B. (1994) *J. Mol. Biol. 235*, 1271−1277.

Kennedy, S. P., Warren, S. L., Forget, B. G., & Morrow, J. S. (1991) *J. Cell Biol. 115*, 267−277.

Kennedy, S. P., Weed, S. A., Forget, B. G., & Morrow, J. S. (1994) *J. Biol. Chem. 269*, 11400−11408.

Kotula, L., DeSilva, T. M., Speicher, D. W., & Curtis, P. J. (1993) *J. Biol. Chem. 268*, 14788−14793.

LaBrake, C. C., Wang, L., Keiderling, T. A., & Fung, L. W.-M. (1993) *Biochemistry 32*, 10296−10302.

Lange, Y., Hadesman, R. A., & Steck, T. L. (1982) *J. Cell Biol. 92*, 714−721.

Laue, T. M., Shah, B. D., Ridgeway, T. M., & Pelletier, S. L. (1992) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., & Horton, J. C., Eds.) pp 90−125, The Royal Society of Chemistry, Cambridge, U.K.

Liu, S. C., Derick, L. H., & Palek, J. (1987) *J. Cell Biol. 104*, 527−536.

Lusitani, D. M., Qtaishat, N., LaBrake, C. C., Yu, R. N., Davis, J., Kelley, M. R., & Fung, L. W.-M. (1994) *J. Biol. Chem. 269*, 25955−25958.

Lux, S. E., & Palek, J. (1995) in *Blood: Principles and practice of hematology* (Handin, R. I., Lux, S. E., & Stossel, T. P., Eds.) pp 1701−1818, J. B. Lippincott Co., Philadelphia.

Mikkelsen, A., & Elgsaeter, A. (1978) *Biochim. Biophys. Acta 536*, 245−251.

Mikkelsen, A., Stokke, B. T., & Elgsaeter, A. (1984) *Biochim. Biophys. Acta 786*, 95−102.

Ralston, G., Cronin, T. J., & Branton, D. (1996) *Biochemistry 35*, 5257−5263.

Sahr, K. E., Laurila, P., Kotula, L., Scarpa, A. L., Coupal, E., Leto, T. L., Linnenbach, A. J., Winkelmann, J. C., Speicher, D. W., Marchesi, V. T., Curtis, P. J., & Forget, B. G. (1990) *J. Biol. Chem. 265*, 4434−4443.

Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.

Shen, B. W., Josephs, R., & Steck, T. L. (1986) *J. Cell Biol. 102*, 997−1006.

Shotton, D. M., Burke, B. E., & Branton, D. (1979) *J. Mol. Biol. 131*, 303−329.

Speicher, D. W., & Marchesi, V. T. (1984) *Nature 311*, 177−180.

Speicher, D. W., Weglarz, L., & DeSilva, T. M. (1992) *J. Biol. Chem. 267*, 14775−14782.

Speicher, D. W., DeSilva, T. M., Speicher, K. D., Ursitti, J. A., Hembach, P., & Weglarz, L. (1993) *J. Biol. Chem. 268*, 4227−4235.

Svoboda, K., Schmidt, C. F., Branton, D., & Block, S. M. (1992) *Biophys. J. 63*, 784−793.

Tse, W. T., Lecomte, M.-C., Costa, F. F., Garbarz, M., Feo, C., Boivin, P., Dhermy, D., & Forget, B. G. (1990) *J. Clin. Invest. 86*, 909−916.

Ursitti, J. A., Kotula, L., DeSilva, T. M., Curtis, P. J., & Speicher, D. W. (1996) *J. Biol. Chem. 271*, 6636−6644.

Vertessy, B. G., & Steck, T. L. (1989) *Biophys. J. 55*, 255−262.

Winkelmann, J. C., Chang, J. G., Tse, W. T., Scarpa, A. L., Marchesi, V. T., & Forget, B. G. (1990) *J. Biol. Chem. 265*, 11827−11832.

Winograd, E., Hume, D., & Branton, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10788−10791.

Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C., & Branton, D. (1993) *Science 262*, 2027−2030.

Yoshino, H., & Marchesi, V. T. (1984) *J. Biol. Chem. 259*, 4496−4500.

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