αβ Spectrin Coiled Coil Association at the Tetramerization Site†

Shahila Mehboob, Bing-Hao Luo, Biraj M. Patel, and L. W.-M. Fung*

Department of Chemistry, Loyola University of Chicago, 6525 N. Sheridan Road, Chicago, Illinois 60626

Received May 14, 2001; Revised Manuscript Received August 13, 2001

ABSTRACT: On the basis of sequence homology studies, it has been suggested that the association of human erythrocytes α and β spectrin at the tetramerization site involves interactions between helices. However, no empirical details are available, presumably due to the experimental difficulties in studying spectrin molecules because of its size and/or its structural flexibility. It has been speculated that erythrocyte tetramerization involves helical bundling rather than coiled coil association. We have used recombinant spectrin peptides to model α and β spectrin to study their association at the tetramerization site. Two α peptides, Spα1−156 and Spα1−368, and one β peptide, Spβ1898−2083, were used as model peptides to demonstrate the formation of the αβ complex. We also found that the replacement of R28 in Spα1−368 to give Spα1−368R28C abolished complex formation with the β peptide. Circular dichroism techniques were used to monitor the secondary structures of the individual peptides and of the complex, and the results showed that both Spα1−156 and Spβ1898−2083 peptides in solution, separately, included helices that were not paired with other helices in the absence of their binding partners. However, in a mixture of Spα1−156 and Spβ1898−2083 and formation of the αβ complex, the unpaired helices associated to form coiled coils. Since the sequences of these two peptides that are involved in the coiled coil association are derived from a native protein, the information obtained from this study also provides insight toward a better understanding of naturally occurring coiled coil subunit-subunit association.

Sequence homology studies of spectrin from human erythrocytes suggest that its αβ subunit association at the tetramerization site involves helical bundling (*1*−*4*). Since the sequences of the suggested helices at the tetramerization site exhibit a general pattern of heptad repeats found in coiled coils, it is possible that the suggested helical bundling at the tetramerization site involves coiled coil subunit−subunit association. The α-helical coiled coil interaction is one of the most common, and probably the simplest, subunit−subunit association in proteins (*5*, *6*). However, the three-dimensional structures of erythrocyte spectrin are not known. Spectrin is a protein ubiquitous among vertebrate tissues and has been identified in a variety of organisms (*7*−*11*). It is believed that all spectrin molecules share certain general properties such as structural domain conformation, and yet, each exhibits specific properties that are important for its specific functions. Thus, structural and functional studies of human erythrocyte spectrin at a molecular/atomic level will improve our understanding not only of erythrocyte spectrin but also of spectrin isoforms as well as coiled coil subunit−subunit association in native proteins.

Both α and β subunits of human erythrocyte spectrin consist of multiple homologous sequence motifs, with each motif presumably folding into a three-helix coiled coil domain with a structure similar to the structures determined for *Drosophila* spectrin (*12*) and chicken brain spectrin (*13*, *14*). α and β spectrin associate at the N-terminal end of the β and the C-terminal end of the α subunit (dimer nucleation site) with high affinity (nanomolar K_d values)[1] to give αβ hetero-dimers (*15*, *16*). It has been suggested that spectrin dimers associate to form spectrin tetramers, with association site at the other end of the dimers, involving two sets of identical, low-affinity (micromolar K_d values) interactions between the N-terminal region of the α subunit (αN) of one αβ dimer and the C-terminal region of the β subunit (βC) in another αβ dimer to give an $(\alpha\beta)_2$ tetramer (*2*, *3*, *17*, *18*). Sequence homology studies predict that about 30 residues at this αN region, prior to the first structural domain, fold into α helical conformation; likewise, about 60 residues in the βC region, following the last structural domain, fold into two helices (*2*−*4*). These one or two helical regions are termed partial domains. Thus, it is logical to assume that the dimer to tetramer formation involves interactions between these partial domains (*2*−*4*, *18*−*20*), and it has been speculated, based on chicken brain spectrin studies, that erythrocyte spectrin tetramerization involves helical bundling, but not coiled coil association (*13*). However, there are little experimental data to substantiate the structures predicted by sequence homology studies in human erythrocyte spectrin or to compare the erythrocyte spectrin structure with the structures from *Drosophila* or chicken brain spectrin. The αN/βC association in spectrin is currently not well understood. Yet, mutations affecting this association lead to

† This work was supported by an NSF grant to L.W.-M.F. (MCB 9801870) and by predoctoral fellowships from the American Heart Association, Chicago Affiliate, to S.M. (9910101Z) and to B.H.L. (0010153Z).

* To whom all correspondence should be addressed. Phone: (773) 508-3128. Fax: (773) 508-3086. E-mail: lfung@luc.edu.

[1] Abbreviations: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; GST, glutathione-S-transferase; IC_{50}, concentration of unlabeled peptide needed to inhibit/displace 50% of labeled peptide from binding to its partner; K_d, dissociation constant; kDa, kilodaltons; R_f, relative front; TFE, 2,2,2-trifluroethanol.

10.1021/bi010984k CCC: $20.00
Published on Web 09/22/2001

abnormal erythrocytes (*9*). In addition, brain spectrin appears to exhibit αN/βC interactions that differ from those found in erythrocyte spectrin (*18*).

We have prepared a number of recombinant α spectrin model peptides of different sizes, with sequences from the αN-terminal region of erythrocyte spectrin, and have shown that recombinant peptides with multiple domains are more stable than the peptides with a single domain (*21*, *22*). Others have also published similar findings (*23*, *24*). However, the smaller peptide, Spα1−156 (a peptide with a sequence consisting of the first 156 residues in α spectrin) associates with β spectrin just as well as the larger peptide, Spα1−368 (a peptide with a sequence consisting of the first 368 residues) (*20*). Both peptides associate with β spectrin isolated from human red blood cells in a manner very similar to that found in intact α and β spectrin, with IC_{50} values of 0.2−0.3 μM. Thus, both Spα1−156 and Spα1−368 can be used as α spectrin model peptides for functional studies. In this study, we also prepared a model β peptide, Spβ1898−2083 (a peptide with the sequence of residues 1898−2083 in β spectrin), and found that it associated with the α spectrin model peptides at a micromolar concentration range. Thus, Spβ1898−2083 is a functional model peptide for β spectrin. We used these α and β model peptides to study their association with each other in order to understand α and β spectrin association at the tetramerization region. Our circular dichroism data provided experimental evidence to suggest that their association involved coiled coil interactions. A peptide that appears to be similar to Spβ1898−2083 has been used by others (*19*, *25*) under somewhat different buffer conditions, and some of their conclusions differ from ours, as discussed below. Since our peptides, and thus the helices involved in coiled coils interaction, were derived from sequences of a native protein, this study will also provide information for coiled coils interaction in other native proteins. In native proteins, the heptad repeat sequences in the helical region may not necessarily follow the critically matched hydrophobic and hydrophilic sequence patterns and interactions (*26*) found in many de novo designed peptides used to study coiled coil association (*6*, *27*−*30*). The interactions between these "nonideal" but native coiled coils are not as well studied as those in de novo designed peptides, yet these interactions may be functionally significant. For example, relatively unstable coiled coil interactions have been found to be essential for optimal mechanical performance of smooth muscle myosin (*31*).

MATERIALS AND METHODS

Spectrin Recombinant Peptides. The peptides Spα1−156, Spα1−368, and Spα1−368R28C were prepared as before (*20*). The three cysteine residues at positions 167, 224, and 324 in Spα1−368R28C were replaced with alanine. For the β spectrin peptide, the cDNA encoding the C-terminal region of the β spectrin subunit was used as a template for the polymerase chain reaction, using *Pfu* (Stratagene, LaJolla, CA) as the DNA polymerase. A primer containing the sequence corresponding to residues 1898−2008 in the β spectrin, in a sense orientation, and a primer containing the sequence corresponding to residues 2073−2083, in an antisense orientation, were used to provide the DNA fragment for a peptide with the sequence of β spectrin from residues 1898 to 2083 (Spβ1898−2083). The DNA fragment was ligated into the *Bam*HI and *Eco*RI sites of a modified glutathione-S-transferase (GST) expression vector pGEX-2T (Amersham Pharmacia Biotech, Piscataway, NJ).

Spα1−156, Spα1−368, Spα1−368R28C, and Spβ1898−2083 were cleaved by thrombin from GST fusion proteins following standard laboratory methods (*32*). As part of the thrombin recognition sequence, Gly-Ser remained as the first two residues in all peptides after thrombin cleavage. Peptide identity and purity were checked by polyacrylamide gel electrophoresis and mass spectrometry using electrospray ionization techniques. Protein concentrations were determined with absorbance values at 280 nm, using extinction coefficient values determined from the primary sequence (http://www.expasy.ch/tools/protparam.html; 16 500 cm^{-1} M^{-1} for Spα1−156, 50 070 cm^{-1} M^{-1} for Spα1−368 and Spα1−368R28C and 31 010 $cm^{-1}M^{-1}$ for Spβ1898−2083).

Spα1−156/Spβ1898−2083 Complex. Our standard solid-phase assay using ^{125}I-labeled peptides was applied to study the affinities between Spα1−156 and Spβ1898−2083, following our published methods (*20*). In addition, to monitor the peptide association qualitatively, native polyacrylamide (6%) gel electrophoresis of Spα1−156, Spα1−368, Spα1−368R28C, and Spβ1898−2083 samples and of their mixtures (with about 25 μM α peptide and 50 μM β peptide, incubated for 16 h at 4 °C) was carried out in a 40 mM Tris buffer with 20 mM sodium acetate and 2 mM EDTA at pH 7.4, following published procedures (*33*), with the following modifications. The slab gel dimension was 72 × 100 mm and 0.5 mm thick, and the gels were run at 100 V for only about 70 min at either 4 or 25 °C, with a change of buffer when the bands were halfway to prevent pH drift.

In our previous studies of binding affinities using ^{125}I solid-phase assay (*20*), we have found that, for the association of Spα1−368 and β spectrin, the t_{on} was found to be about 10 h^{-1}, and t_{off} was >6 h for the majority (80%) of the sample. t_{off} for the other 20% was ∼10 min (*20*). Thus, our standard incubation time of α and β peptides was 16 h, assuming that the β peptide is similar to β spectrin, and the electrophoresis was done in a time interval shorter than 6 h.

Circular Dichroism. Circular dichroism (CD) samples of Spα1−156 (about 10 μM) and Spβ1898−2083 (about 10 μM) peptides and of their equimolar mixtures (each about 10 μM) were in 5 mM phosphate buffer with 150 mM NaCl at pH 7.4 (PBS7.4). All samples with and without either 60% 2,2,2-trifluroethanol (TFE, from Fischer Biotech) or different concentrations (0−6 M) of urea (Fischer Biotech) were incubated at 4 °C for about 16 h prior to CD measurements.

CD measurements were performed on a JASCO 710 CD spectrometer, using a thermostated cell with 0.1 cm path length either at 25 °C or from 25 to 40 °C with a 2 °C increment and from 40 to 80 °C with a 5 °C increment. The spectra were obtained at 0.5 nm resolution from 190 to 250 nm. Spectra of PBS7.4 buffer under similar CD conditions were used to correct spectral baselines of the samples. Ellipticity (θ, deg) values from CD spectra were converted to molar residue ellipticity ($[\theta]$, deg cm^2 $dmol^{-1}$) values. Helical contents were calculated from values of the amide $n\pi^*$ transition at 222 nm ($[\theta_{222}]$), using a value of −36 000 deg cm^2 $dmol^{-1}$ to represent 100% α helical content (*34*−*36*).

Molar residue ellipticity values of the $\pi\pi^*$ transition at 208 nm ($[\theta_{208}]$) were also calculated, and the $[\theta_{222}]/[\theta_{208}]$ ratios were obtained.

RESULTS

Peptide Characterization. SDS−PAGE results indicated that the peptides Spα1−156 (with electrophoretic mobility corresponding to a molecular mass of ∼17 kDa), Spα1−368 and Spα1−368R28C (∼43 kDa), and Spβ1898−2083 (∼22 kDa) were at least 95% pure. Molecular masses from electrospray ionization mass spectrometry were 18.67 kDa for Spα1−156, 42.93 kDa for Spα1−368, 42.80 kDa for Spα1−368R28C, and 22.04 kDa for Spβ1898−2083 and were within 0.1% of the theoretical molecular masses (18.67, 42.94, 42.84, and 22.04 kDa, respectively). Spα1−156 and Spα1−368 and Spα1−368R28C, but not Spβ1898−2083, have been characterized previously (*20*). For Spβ1898−2083, the DNA fragment inserted into the pGEX-2T vector was sequenced to confirm the positions of the start and stop codons and also the entire sequence of the peptide. The α helical content from CD analysis of Spβ1898−2083 was ∼55%. This value was similar to Spα1−156 (*20*) as well as to those obtained for other spectrin peptides of similar size (*37*, *38*), suggesting that the Spβ1898−2083 peptide was also well folded.

Spα1−156/Spβ1898−2083 Complex. The ^{125}I solid-phase assay yielded a mean IC_{50} value of 0.14 ± 0.04 μM for the Spα1−156/Spβ1898−2083 complex. The IC_{50} values obtained for Spα1−156 and Spα1−368 with intact β spectrin was about 0.3 μM (*20*).

Native gel electrophoresis data at 4 °C (Figure 1) showed the migration pattern of samples containing Spα1−156 (lane 1), Spα1−368 (lane 2), and Spβ1898−2083 (lane 3) peptides, with R_f values of 0.52, 0.52, and 0.27, respectively. The mass-to-charge ratios obtained from sequence information were similar for Spα1−156 (1.7) and Spα1−368 (2.3) but larger for Spβ1898−2083 (4.4). Thus, it was not surprising to find that the measured R_f values for Spα1−156 and Spα1−368 bands were similar and were larger than that of Spβ1898−2083.

In mixture samples with limiting α peptide concentrations (containing about 25 μM α and 50 μM β peptides), the band corresponding to the α peptide disappeared (either with Spα1−156 as the α peptide in lane 4, or with Spα1−368 as the α peptide in lane 5), and only one band with R_f values of 0.31−0.32 appeared, suggesting that this was the band for the αβ complex and the nonreacting β peptide in the mixtures. Results of gels run at 4 and 25 °C were similar. Since the concentration of the α peptide was limited in the mixture samples, about 99% (24.75 μM) of the α peptides was estimated to be in the associated state, using the IC_{50} value of 0.14 μM that we obtained as the K_d value for αβ complex dissociation.

Although we have previously shown that peptides with more than one 106-amino-acid-sequence motif are needed to mimic the structural stability of spectrin (*21*, *22*), these results suggested that Spα1−156 associated with Spβ1898−2083 in a manner very similar to that of Spα1−368. Thus, either Spα1−156 or Spα1−368 could be used as a model peptide for α spectrin to study its association with β spectrin at the tetramerization site.

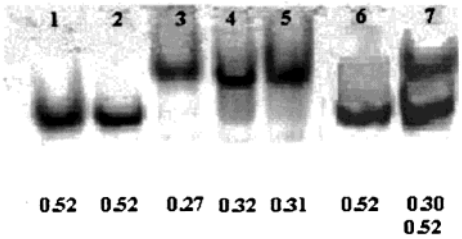

FIGURE 1: Native polyacrylamide gel (6%) electrophoresis of spectrin peptides, each sample with 4 μg of protein(s): Spα1−156 (lane 1), Spα1−368 (lane 2), Spβ1898−2083 (lane 3), Spα1−156/Spβ1898−2083 at a 1:2 molar ratio (22.0 μM/44.3 μM) (lane 4), Spα1−368/Spβ1898−2083 at a 1:2 molar ratio (24.4 μM /48.8 μM) (lane 5), Spα1−368R28C (lane 6), and Spα1−368R28C/Spβ1898−2083 at a 1:2 molar ratio (25/50 μM, or about 2 μg each) (lane 7). All samples were incubated at 4 °C for 16 h. Gel running buffer contained 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA at pH 7.4. Prior to loading, 4 μg of each sample was mixed with 1 μL of the loading buffer (7.75 mL of gel running buffer and 1.25 mL of 1% solution of bromophenol blue, 3.5 mL of water and 12.5 mL of glycerol). The gel was run at 4 °C with 100 V for 70 min, with a change of buffer when the bands were halfway to prevent pH drift. The gels were then soaked in a fixing solution (40% methanol, 10% acetic acid, and 0.01% bromophenol blue), for 30 min, and then transferred to a staining solution (10% acetic acid, 0.01% bromophenol blue) and soaked for 60 min. Before visualizing, the gels were destained in 7% acetic acid for 30 min. The total amounts of protein loaded in each lane were all about 4 μg. R_f values are shown at the bottom of the gel. The emergence of bands with new R_f values in lanes 4 and 5 demonstrates the association of α and β spectrin peptides in the mixtures (αβ complex). The appearance of the α peptide band with an R_f value of 0.52 in lane 7 shows that the mutant peptide (Spα1−368R28C) did not associate with the β spectrin peptide under these concentration conditions.

Similar α and β peptide mixtures were prepared with Spα1−368 replaced with Spα1−368R28C. Two separate bands with R_f values of 0.30 and 0.52 were observed in this mixture (lane 7). Thus, no association between α and β peptides was observed for Spα1−368R28C and Spβ1898−2083 at a micromolar concentration range. Since Spα1−368R28C had three intrinsic cysteine residues replaced with alanine residues, we also replaced these three cysteine residues in Spα1−368 with alanine residues and found that this cysteine-less peptide and Spα1−368 associated with the β peptide in a similar manner (data not shown). Thus, the lack of association of Spα1−368R28C with Spβ1898−2083 was not due to the removal of cysteine residues in the structural domains but due to a specific replacement of arginine at position 28 with a cysteine residue in Spα1−368.

CD Studies of Spα1−156 and Spβ1898−2083 Peptides. The CD spectra of Spα1−156 and Spβ1898−2083 peptides exhibited characteristic features of α helices, with minima at 222 and 208 nm (Figure 2).

The amide $\pi\pi^*$ transition at 208 nm is sensitive to inter-helix coupling, resulting in a decrease in the values of $[\theta_{208}]$, whereas the $n\pi^*$ transition exhibits only a weak short-range coupling, resulting in only a small change in the values of $[\theta_{222}]$ in the presence of inter-helix coupling (*39*). Empirical studies show that coiled coil systems generally exhibit $[\theta_{222}]/[\theta_{208}]$ values around 1 or greater than 1, whereas nonassociated helices exhibit values around 0.8−0.9 (*35*, *40*). Thus, the $[\theta_{222}]/[\theta_{208}]$ values have been widely used to distinguish between associated coiled coil helices and nonassociated

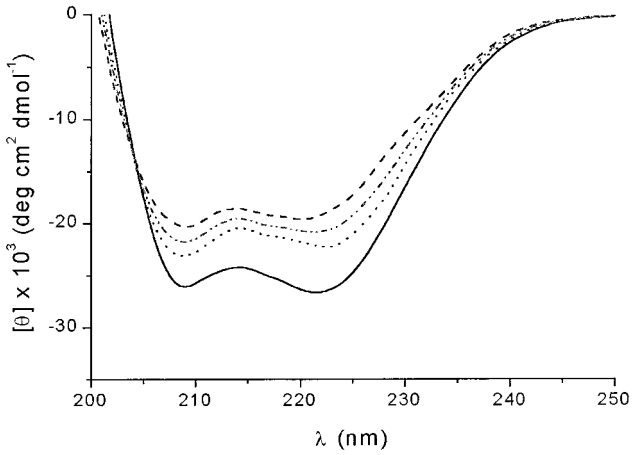

FIGURE 2: Typical CD spectra of Spα1−156 (dash), Spβ1898−2083 (dot), and a mixture of Spα1−156 and Spβ1898−2083 in PBS 7.4 buffer (solid). The CD spectra were recorded at room temperature (23 °C). The peptide concentrations in all samples were about 12 μM. The mathematical addition of individual spectra of Spα1−156 and Spβ1898−2083 (− · · −) is also shown.

Table 1: Mean Residue Molar Ellipticity Ratios at 222 and 208 nm Are Shown for Spα1−156, Spβ1898−2083, and for the $\alpha\beta$ Complex, in the Absence and Presence of 60% TFE[a]

	$[\theta_{222}]/[\theta_{208}]$		
	no TFE		60% TFE
peptide	observed[a]	estimated[b]	observed[c]
Spα1−156	0.93 ± 0.03	0.95	0.88 ± 0.01
Spβ1898−2083	0.95 ± 0.03	0.92	0.89 ± 0.01
Spα1−156/Spβ1898−2083 complex	1.03 ± 0.03	1.00	0.88 ± 0.01

[a] The mean peptide concentration was 10 ± 2 μM. The CD spectra were recorded at room temperature (23 °C) in PBS 7.4 buffer. [a] Mean values ± standard deviation values (s_{n-1}), $n = 7$. [b] Estimated values were obtained by assuming a value of 1.0 for associated coiled coils and 0.8 for nonassociated helices. We considered Spα1−156 to consist of 75% associated coiled coils and 25% unassociated helices and Spβ1898−2083 to consist of 60% associated coiled coils and 40% nonassociated helices. [c] Mean values ± standard deviation values (s_{n-1}), $n = 3$.

helices (*35*, *36*, *39*−*42*), although this application has been questioned (*43*, *44*). It has been shown that some systems, such as poly (Glu) and poly (Lys) (*44*) or a random copolymer of Lys, Glu, and Ala (*45*) that do not form coiled coils have $[\theta_{222}]/[\theta_{208}] > 1$. Other factors, such as helix geometry and solvent, may also affect the ratio.

The mean $[\theta_{222}]/[\theta_{208}]$ value obtained from the CD spectra of Spα1−156 was 0.93 ± 0.03 ($n = 7$) (Table 1). This value suggested the presence of one or more nonassociated helices in Spα1−156. Our previous NMR data show that this peptide consists of four helices (*46*). If we assume that three helices (75%) are associated as coiled coils, with $[\theta_{222}]/[\theta_{208}]$ values of 1, and one helix (25%) is nonassociated with a $[\theta_{222}]/[\theta_{208}]$ value of 0.8, we calculated a $[\theta_{222}]/[\theta_{208}]$ value of 0.95 for the molecule.

For Spβ1898−2083 peptide, the mean $[\theta_{222}]/[\theta_{208}]$ value obtained from the CD spectra was 0.95 ± 0.03 ($n = 7$), again suggesting the presence of nonassociated helix/helices.

CD Studies of Spα1−156/Spβ1898−2083 Complex. (1) Coiled Coils Association. (a) $[\theta_{222}]/[\theta_{208}]$ Value. It is interesting to note that the spectra of samples containing a mixture of α and β peptides differed from the weighted sum of individual α and β spectra (Figure 2). If the α and β peptides remained as individual peptides in the mixture, the $[\theta_{222}]/[\theta_{208}]$ value would be the average value of those of α and β peptides, i.e. about 0.94. However, the mean value obtained from the spectra of the mixture was 1.03 ± 0.03 ($n = 7$). A statistical *t*-test (one tail and two sample unequal variance) for the seven data sets for Spα1−156 samples and for Spα1−156/Spβ1898−2083 mixture samples yielded a probability (*p*) of <0.001 of being the same (*t*-test result = 2.8×10^{-5}). Similarly, the result of a *t*-test for Spβ1898−2083 samples and Spα1−156/Spβ1898−2083 mixture samples was 5.7×10^{-5}. Thus the mean value of the mixture samples (1.03) in Table 1 was significantly different than the mean values of either α (0.93) or β (0.95) peptides. When we applied the *t*-tests to the data for Spα1−156 and for Spβ1898−2083, we found that the two sets of values were similar, with a *t*-test result of 0.1. Using the results of solid-phase assays (K_d value of 0.14 μM) for the Spα1−156/Spβ1898−2083 system consisting of 10 μM of each peptide, we expect about 89% of Spα1−156 and Spβ1898−2083 to be in the associated state. Thus, the CD spectra of the mixtures were mostly of Spα1−156/Spβ1898−2083 complex.

Since the coiled coil systems generally exhibit $[\theta_{222}]/[\theta_{208}]$ values around 1 or greater than 1 (*35*, *40*), a $[\theta_{222}]/[\theta_{208}]$ value of 1.03 suggests that all helices in the mixture samples are associated as in coiled coil systems.

We recognize that the values of the ratio alone does not uniquely suggest coiled coil formation. However, taking other information such as the heptad sequence motif in spectrin peptides and the NMR structure of Spα1−156 into consideration, the suggestion of coiled coil formation is a simple and a very likely one.

However, in 60% TFE, the $[\theta_{222}]/[\theta_{208}]$ values of the mixture samples were 0.89 ± 0.01, similar to the values obtained for samples containing only Spα1−156 or only Spβ1898−2083 in TFE (Table 1). Since $[\theta_{222}]/[\theta_{208}]$ values around 0.8−0.9 suggest nonassociated helices (*35*, *40*), these results suggested that helices in coiled coil conformation in the $\alpha\beta$ complex as well as the structural domain helices in individual peptides dissociated into noninteracting helices in 60% TFE. Since TFE is a solvent known to interfere with hydrophobic interactions (*36*, *40*) and to affect hydrogen bonding and solvent structure (*47*), these results suggested that either the helices associated via a hydrophobic effect to form structural domains as well as the $\alpha\beta$ complex, or TFE induced other conformational changes in our system. In general, conformational changes effected by TFE appear to depend on the particular amino acid sequences, the TFE concentration and other solution conditions as well as on the structures involved (*47*).

(b) Helical Contents. The values of $[\theta_{222}]$ are often used to monitor secondary structures, as they are sensitive to the helicity of peptides (*34*, *40*). The mean value was -18.9×10^3 deg cm^2 dmol^{-1}, corresponding to α helical content of ∼52%, for Spα1−156, and -20.6×10^3 deg cm^2 dmol^{-1} (∼57%) for Spβ1898−2083. The mean value of $[\theta_{222}]$ for Spα1−156/Spβ1898−2083 complex was $(-25.4 \pm 2.1) \times 10^3$ deg cm^2 dmol^{-1}, corresponding to α helical content of ∼70% (Table 2, Figure 2). This helical content value for the complex was much higher than the weighted sum

Table 2: $[\theta_{222}]$ and the Helicity (%) for Spα1−156, Spβ1898−2083, and the αβ Complex in the Absence and Presence of 60 % TFE[a]

peptide	no TFE[a]		60% TFE[b]	
	$[\theta_{222}] \times 10^3$ (deg cm^2 dmol^{-1})	helical content[c] (%)	$[\theta_{222}] \times 10^3$ (deg cm^2 dmol^{-1})	helical content[c] (%)
Spα1−156	-18.9 ± 1.7	52 ± 5	-28.7 ± 5.3	80 ± 15
Spβ1898−2083	-20.6 ± 1.4	57 ± 4	-32.8 ± 5.2	91 ± 15
Spα1−156/Spβ1898−2083 complex	-25.4 ± 2.1	70 ± 6	-32.0 ± 3.7	89 ± 10

[a] CD spectra were recorded at room temperature (23 °C), in PBS 7.4 buffer. The peptide concentrations were 10 ± 2 μM. [a] Mean values ± standard deviation values (s_{n-1}), $n = 7$. [b] Mean values ± standard deviation values (s_{n-1}), $n = 3$. [c] Helical content values were calculated using a $[\theta_{222}]$ value of -36×10^3 deg cm^2 dmol^{-1} to represent 100% a helix.

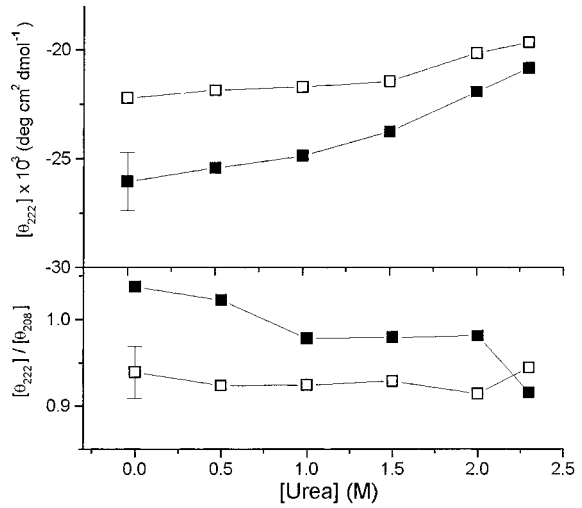

FIGURE 3: Mean $[\theta_{222}]$ values and the mean $[\theta_{222}]/[\theta_{208}]$ ratio of the mixture containing Spα1−156 (10 μM) and Spβ1898−2083 (10 μM) in PBS 7.4 buffer with different concentrations of urea (solid squares) and the values of the weighted sum of Spα 1−156 and Spβ1898−2083 (open squares) obtained under similar conditions. The standard deviation values (s_{n-1}) were plotted as error bars ($n = 2$).

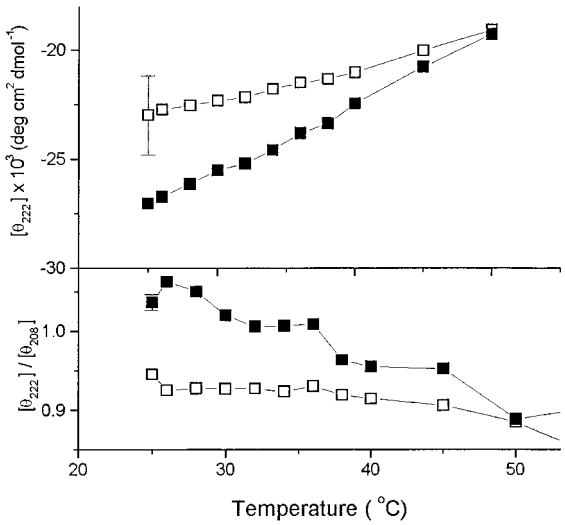

FIGURE 4: Mean $[\theta_{222}]$ values and the mean $[\theta_{222}]/[\theta_{208}]$ values of the mixture containing Spα1−156 and Spβ1898−2083 (solid squares) and the values of the weighted sum of those from Spα 1−156 and Spβ1898−2083 peptides (open squares) at different temperatures are shown. The CD spectra were recorded in PBS 7.4 buffer. The standard deviation values (s_{n-1}) ($n = 2$) were plotted as error bars.

(∼55%) of Spα1−156 and Spβ1898−2083 peptides. Thus, an apparent helical content increase of about 10−15% accompanied complex formation in Spα1−156 and Spβ1898−2083 peptides.

We also found that, in 60% TFE, the helicity of all samples (samples consisting of individual peptides as well as of the complex) increased 20−30% (Table 2). TFE has been shown to increase the helicity of many helical peptides (*36*, *48*) but to decrease the helicity in some peptides with very high helical content (*48*).

(2) Urea and Thermal Denaturation Studies on the Spα1−156/Spβ1898−2083 Complex. The values of $[\theta_{222}]/[\theta_{208}]$ and of $[\theta_{222}]$ of the αβ complex without urea were higher than the weighted sum of individual Spα1−156 and Spβ1898−2083 peptides (Figure 3). However, upon addition of urea, the differences in the values decreased upon increasing urea concentrations, and the two values merged to a similar value at a urea concentration of 2 M.

Similar results were observed upon increasing the temperature of the samples (Figure 4). The difference between samples containing a mixture of α and β peptides and samples containing individual α or β peptides narrowed as the temperatures increased from 25 to 50 °C. These results indicated that the increase in helical content in the αβ complex correlated well with αβ association. When the association was disrupted either by urea or by temperature denaturation, the $[\theta_{222}]/[\theta_{208}]$ values and the helical contents of the αβ mixture became similar to those of α and β peptides.

DISCUSSION

Molecular Structure for Spα1−156. Detailed three-dimensional structures are not presently available for either Spα1−156 or Spβ1898−2083. However, preliminary NMR studies of Spα1−156 (*46*) identified four α-helices, consisting of residues 21−45, 53−81, 88−118, and 123−153, as well as five random coil regions, consisting of residues 1−20, 46−52, 82−87, 119−122 and 154−156. In addition, NMR data from spin-labeled Spα1−156 suggest that the first helix does not bundle with the other three helices, but appears to be a lone helix (*46*). Since we identify the first residue in the structural domain of α spectrin as residue 52 (*32*), we assume that the remaining three helices, consisting of residues 53−81, 88−118, and 123−153, interact to form a triple helical coiled coil structural domain, generally similar to those found in *Drosophila* spectrin (*12*) and in chicken brain spectrin (*13*, *14*), but with specific differences, such as helical lengths (*46*). Thus, it appears that the lone helix in Spα1−156 observed by NMR, consisting of residues 21−45 prior to the first structural domain, is responsible for the $[\theta_{222}]/[\theta_{208}]$ values to be less than one obtained from our CD data for Spα1−156. The sequence of this nonassociated helix is shown in Table 3.

Table 3: Sequence Assignments of Helices and Heptads of Erythrocyte Spectrin Fragments in the αN and βC Regions

αN and βC Regions.

α spectrin	Residues 21 - 45 (Helix C)	AEEIQERRQEVLTRYQSFKERVAER
	Heptad repeat	*abcdefgabcdefgabcdefgabcd*
β spectrin	Residues 2008 - 2037 (Helix A)	LLEVCQFSRDASVAEAWLIAQEPYLASGDF
	Heptad repeat	*efgabcdefgabcdefgabcdefgabcdef*
	Residues 2044 - 2072 (Helix B)	VEKLIKRHEAFEKSTASWAERFAALEKPT
	Heptad repeat	*abcdefgabcdefgabcdefgabcdefga*

Working Model for Spβ1898-2083. Since no structural information is available for Spβ1898−2083, we suggested the following working model structure for this peptide. On the basis of sequence homology and assuming that the tryptophan at residue 2024 is the conserved tryptophan in the spectrin structural domain (*49*, *50*), sequence alignment puts residues 1902−2007 as the 106-amino-acid motif, which presumably folds into a triple helical coiled coil domain structure in Spβ1898−2083. Guided by heptad patterns in the sequence, we further assume (a) that the region following this structural domain consisting of residues 2008−2083 forms two helices, with residues 2008−2037 similar to residues 53−81 (helix A) in Spα1−156 and residues 2044−2072 similar to residues 88−118 (helix B) in Spα1−156 (Table 3), and (b) that these two helices do not associate among themselves or with the structural domain in the absence of its α partner. Thus, this working model for Spβ1898−2083 consists of three (60%) associated coiled coil helices, and two (40%) nonassociated helices, with an estimated $[\theta_{222}]/[\theta_{208}]$ value of 0.92, a value similar to our experimental value of 0.95 ± 0.03. If Spβ1898−2083 has associated helices in the structural domain and no nonassociated helices in the partial domain region, then the expected $[\theta_{222}]/[\theta_{208}]$ value would be one, or larger than one. Our suggestion that, in the absence of α spectrin, the C-terminal region of β spectrin consists of two nonassociated helices differs from an earlier suggestion that this region is unstructured in the absence of its α partner (*25*). This difference may be due to different buffer conditions used in the two studies. The actual structural information for Spβ1898−2083 awaits experimental data. Our working model is used merely to provide possible interpretations to our data.

αN/βC Association in Spectrin. As demonstrated by the IC_{50} values and by our native gel electrophoresis data, Spα1−156 and Spβ1898−2083 associate in the micromolar concentration range in a manner similar to α and β spectrin association at the tetramerization site. Thus, Spα1−156 and Spβ1898−2083 are good model peptides for studying the αN/βC association in spectrin. However, these binding results do not provide any information on the sites involved in α and β peptide association. Using our working models for both peptides, the possible sites of association for these two peptides are (a) between the coiled coil structural domains of α and β peptides, to give an association similar to the domain−domain association involving electrostatic interactions between domains found in the dimer nucleation site (*16*), or (b) between the nonassociated helices in the α and β peptides to give coiled coil subunit−subunit association.

The simplest interpretation of our CD results is that the two peptides do not associate via structural domain−structural domain interaction such that the nonassociated helices remain nonassociated, but via an association of nonassociated helices (a single helix of residues 21−45 in Spα1−156 and two helices of residues 2008−2083 in Spβ1898−2083). The association appears to involve hydrophobic effects since it is disrupted by TFE. It is also possible that TFE induced major conformational changes in α and β peptides to disrupt their association.

This association is accompanied by a 15% increase in helicity, similar to an earlier observation (*25*), suggesting stabilization of the nonassociated helices in the α and β peptides upon association. The origin of this increase in helicity is not clear from our current studies. It is possible that some less structured regions in Spα1−156 and in Spβ1898−2083 become more helical upon complex formation. However, the helicity values for both Spα1−156 (about 52%) and Spβ1898−2083 (about 57%) peptides are lower than those for the larger peptides, such as Spα52−262 (α peptide with two structural domains, 83%) and Spα52−368 (α peptide with three structural domains, 76%) (*21*). In a study of amphipathic α-helical peptides, Lazo and Downing (*36*) suggest that peptides with relatively high helical content form a "tight" amphipathic helix with minimal distortion of the backbone hydrogen bonds, whereas peptides with relatively low helical content form a "loose" helix in which the peptide carbonyl groups are tilted outward to give lower mean residue ellipticities. Thus, we speculate that the helices in individual Spα1−156 and Spβ1898−2083 are "loose" helices (*36*) or "frayed" helices (*51*) and thus have lower values for their helicity, whereas the helices in the Spα1−156/Spβ1898−2083 complex as well as those in peptides with more than one structural domains, such as Spα52−368, are "tight" helices and thus have higher values for their helicity. Therefore, the increase in helicity may simply be a property of our model system, and has no mechanistic significance for α and β spectrin association. However, this increase in helicity may be viewed as evidence of coiled coil association between the nonassociated helices, with "frayed" helices converting to "tight" helices upon association. Consequently, the helices in the Spα1−156/Spβ1898−

2083 complex may exhibit conformations that are similar to those in α peptide with three structural domains, such as Spα52−368.

In addition to the hydrophobic effect, the association appears to involve specific charge−charge interactions. Close examination of the sequence of residues 21−45 in Spα1−156 showed a general pattern of *abcdefg* heptad repeats, with residue 21A at an "*a*" position and residue 45R in a "*d*" position (Table 3).

A similar heptad repeat sequence is observed in residues 2008−2037 with residue 2008 (leucine) at an "*e*" position and residues 2044−2072 with residue 2044 (valine) in an "*a*" position in Spβ1898−2083 (Table 3). In Spα1−156, residue 28, although in an "*a*" position, is arginine, not the hydrophobic residues such as leucine or valine usually found at this position (*28*). The replacement of arginine by cysteine at position 28 provides a more hydrophobic residue at this position, and yet such replacement abolishes its ability to associate with Spβ1898−2083 in a micromolar concentration range. Thus, though nonhydrophobic at an "*a*" position, arginine is important in this coiled coil subunit−subunit association (*12*). Using our working model for Spβ1898−2083, we suggest that R28 in α spectrin interacts with a residue having a negatively charged side chain, such as E2069, in β spectrin. A disruption of this charge−charge interaction abolishes αβ association in this region. This suggestion is consistent with clinical mutations found thus far, in that a replacement of arginine with leucine, serine, cysteine or histidine reduces spectrin tetramer concentrations in hereditary elliptocytosis patients (*19*, *52*). In *Drosophila* spectrin domain studies, it has been suggested that the charged end of arginine sticks out of the helix bundle to interact with the neighboring "*e*" position residue, and the base of arginine provides a small hydrophobic surface for hydrophobic packing (*12*). However, the precise nature of this and other interactions in this coiled coil association in human erythrocyte spectrin cannot be deduced from our data presented here. It is interesting to note that residue 45 (arginine) in α spectrin is in a "*d*" position and may also be involved in charge−charge interactions between helices. Future studies showing specific interaction sites are needed to determine interacting components in the coiled coil helices at the tetramerization site of erythrocyte spectrin.

ACKNOWLEDGMENT

We acknowledge the usage of CD facility in Dr. M. E. Johnson's laboratory at the University of Illinois at Chicago with the assistance of Dr. Sunghyouk Park. We thank Dr. B. G. Forget of Yale University for providing us with the spectrin cDNA clone.

REFERENCES

1. Speicher, D. W., and Marchesi, V. T. (1984) *Nature 311*, 177−180.
2. Tse, W. T., Lecomte, M. C., Costa, F. F., Garbarz, M., Feo C., Boivin, P., Dhermy, D., and Forget, B. G. (1990) *J. Clin. Invest. 86*, 909−916.
3. DeSilva, T. M., Peng, K.-C., Speicher, K. D., and Speicher, D. W. (1992) *Biochemistry 31*, 10872−10878.
4. Speicher, D. W., DeSilva, T. M., Speicher, K. D., Ursitti, J. A., Hembach, P., and Weglarz, L. (1993) *J. Biol. Chem. 268*, 4227−4235.
5. Newman, J. R., Wolf, E., and Kim, P. S. (2000) *Proc. Natl. Acad. Sci. 97*, 13203−13208.
6. Burkhard, P., Meier, M., and Lustig, A. (2001) *Protein Sci. 9*, 2294−2301.
7. Gascard, P., and Mohandas, N. (2000) *Curr. Opin. Hematol. 7*, 123−129.
8. DeMatteis, M. A., and Morrow, J. S.(2000) *J. Cell Sci. 113*, 2331−2343.
9. Dhermy, D. (1991) *Biol. Cell. 71*, 249−254.
10. Holleran, E. A., and Holzbaur, E. L. F. (1998) *Trends Cell Biol. 8*, 26−29.
11. Goodman, S. R. (1999) *Brain Res. Bull. 50*, 345−346.
12. Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C., and Branton, D. (1993) *Science 262*, 2027−2030.
13. Pascual, J., Pfuhl, M., Walther, D., Saraste, M., and Nilges, M. (1997) *J. Mol. Biol. 273*, 740−751.
14. Grum, V. L., Li, D., MacDonald, R. I., and Mondragon, A. (1999) *Cell 98*, 523−535.
15. Speicher, D. W., Weglarz, L., and DeSilva, T. M. (1992) *J. Biol. Chem. 267*, 14775−14782.
16. Begg, G. E., Harper, S. L., Morris, M. B., and Speicher, D. W. (2000) *J. Biol. Chem. 275*, 3279−3287.
17. Ralston, G. B. (1991) *Biochemistry 30*, 4179−4186.
18. Begg, G. E., Morris, M. B., and Ralston, G. B. (1997) *Biochemistry 36*, 6977−6985.
19. Nicolas, G., Pedroni, S., Fournier, C., Gautero, H., Craescu, C., Dhermy, D., and Lecomte, M. C. (1998) *Biochem. J. 332*, 81−89.
20. Cherry, L., Menhart, N. M., and Fung, L. W.-M. (1999) *J. Biol. Chem. 274*, 2077−2084.
21. Menhart, N., Mitchell, T., Lusitani, D., Topouzian, N., and Fung, L. W.-M. (1996) *J. Biol. Chem. 271*, 30410−30416.
22. Lusitani, D., Menhart, N. M., Keiderling, T. A., and Fung, L. W.-M. (1998) *Biochemistry 37*, 16546−16554.
23. DeSilva, T. M., Harper, S. L., Kotula, L., Hensley, P., Curtis, P. J., Otvos, Jr., L., and Speicher, D. W. (1997) *Biochemistry 36*, 3991−3997.
24. MacDonald, R. I., and Pozharski E. V. (2001) *Biochemistry 40*, 3974−3984.
25. Lecomte, M. C., Nicolas, G., Dhermy, D., Pinder, J. C., and Gratzer, W. B. (1999) *Eur. Biophys. J. 28*, 208−215.
26. Busson, B., and Doucet, J. (1999) *J. Struct. Biol. 127*, 16−21.
27. Lombardi, A., Bryson, J. W., and Degrado, W. F. (1996) *Biopolymers 40*, 495−504.
28. Wagschal, K., Tripet, B., and Hodges, R. S. (1999) *J. Mol. Biol. 285*, 785−803.
29. Burkhard, P., Strelkov, S. V., and Stetefeld, J. (2001) *Trends Cell Biol. 11*, 82−88.
30. Vu, C., Robblee, J., Werner, K. M., and Fairman, R. (2001) *Protein Sci. 10*, 631−637.
31. Lauzon, A. Fagnant, P., Warshaw, D., and Trybus, K. (2001) *Biophys. J. 80*, 1900−1904.
32. Lusitani, D., Qtaishat, N., LaBrake, C., Yu, R. N., Davis, J., Kelley, M. R., and Fung, L. W.-M. (1994) *J. Biol. Chem. 269*, *42*, 25955−25958.
33. Morrow, J. S., and Marchesi, V. T., (1981) *J. Cell Biol. 88*, 463−468.
34. Greenfield, N., and Fasman, G. D. (1969) *Biochemistry 8*, 4108−4116.
35. Graddis, T. J., Myszka, D. G., and Chaiken, I. M. (1993) *Biochemistry 32*, 12664−12671.
36. Lazo, N. D., and Downing, D. T. (1997) *Biochemistry 36*, 2559−2565.
37. Winograd, E., Hume, D., and Branton, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10788−10791.
38. Kotula, L., DeSilva, T. M., Speicher, D. W., and Curtis P. J. (1993) *J. Biol. Chem. 268* (20), 14788−14793.
39. Cooper, T. M., and Woody, R. Q. (1990) *Biopolymers 30*, 657−676.
40. Lau, S. Y., Taneja, A. K., and Hodges, R. S. (1984) *J. Biol. Chem. 259*, 13253−13261.

41. Shotland, Y., Teff, D., Koby, S., Kobiler, O., and Oppenheim, A. B. (2000) *J. Mol. Biol. 299*, 953−964.
42. Kiss, R. S., Kay, C. M., and Ryan, R. O. (1999) *Biochemistry 38*, 4327−4334.
43. Bode, K. A., and Applequist, J. (1997) *Biopolymers 42*, 855−860.
44. Holtzer, M. E., and Holtzer A. (1995) *Biopolymers 36*, 365−379.
45. Quadrifoglio, F., and Urry, D. W. (1968) *J. Am. Chem. Soc. 90*, 2750−2760.
46. Park, S., Johnson, M. E., and Fung, L. W.-M. (2000) *FEBS Lett. 485*, 81−86.
47. Buck, M. (1998) *Q. Rev. Biophys. 31*, 297−355.
48. Su, J. Y., Hodges, R. S., and Kay, C. M. (1994) *Biochemistry 33*, 15501−15510.
49. 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., and Forget, B. G. (1990) *J. Biol. Chem. 265*, 4434−4443.
50. Winkelmann, J. C., Chang, J. G., Tse, W. T., Scarpa, A. L., Marchesi, V. T., and Forget, B. G. (1990) *J. Biol. Chem. 265*, 11827−11832.
51. Jaravine, V. A., Alexandrescu, A. T., and Grzesiek S. (2001) *Protein Sci. 10*, 943−950.
52. Lecomte, M. C., Garbarz, M., Gautero, H., Bournier, O., Galand, C., Boivin, P., and Dhermy, D. (1993) *Br. J. Haematol. 85*, 584−595.

BI010984K