Purification of Erythrocyte Spectrin α- and β-Subunits at Alkaline pH and Structural and Hydrodynamic Properties of the Isolated Subunits†

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ABSTRACT: A new method for the isolation of the α- and β-subunits of human erythrocyte spectrin was developed, and structural properties and association behavior of the isolated subunits were studied by means of CD, nondenaturing gel electrophoresis, and analytical ultracentrifugation. The α- and β-subunits were isolated using ion-exchange FPLC (pH 11) followed by size-exclusion FPLC (pH 7.5), having shown that alkaline pH dissociates spectrin polymers to their monomers [see Fujita *et al.* (1998) *Biochemistry 37,* 264−271]. The isolated subunits had α-helical content and thermal stability almost equivalent to those of native spectrin and reassembled to form heterodimers and tetramers which were indistinguishable from native spectrin with respect to secondary structure content, thermal stability, migration pattern on nondenaturing gels, and sedimentation coefficients. Thus, our data show that the increase in the structural stability of a heterodimer by association of the two monomers is very small. Sedimentation coefficients for the isolated α- and β-subunits were 6.3 and 5.7 S, respectively. The similar frictional ratios (f/f_0) of the isolated α-subunit (2.42) and the β-subunit (2.45) indicate that the flexibility of both these wormlike chains and the range of shapes they can adopt in solution are very similar. The f/f_0 value for spectrin dimer (2.41) indicates that its flexibility is somewhat, but not grossly, reduced compared to that of the individual subunits. Consequently, the folded repeat units of the subunits and the flexible connections between them are probably "in register" along the length of the dimer.

Erythrocytes undergo elastic deformation and recovery many times during their passage through the circulatory system. This elastic deformability is maintained even after the cell is hemolyzed and the lipid bilayer is stripped in high salt with nonionic detergent such as Triton X-100 (*1*−*4*). The remaining protein meshwork, called the "membrane skeleton" or "shell", is composed largely of spectrin, actin, and protein 4.1 (*2*). Spectrin is the principal component, by weight, of the membrane skeleton and plays a major role in the overall flexibility and elasticity of the erythrocyte membrane (*5*, *6*). Spectrin is comprised of evolutionarily related α-subunits (280 kDa) and β-subunits (246 kDa) (*7*, *8*) which associate in an antiparallel "side-to-side" manner (*9*−*11*) to form an elongated, wormlike heterodimer 100 nm in contour length (*9*). The heterodimers further self-associate in a "head-to-head" fashion to form tetramers, the dominant forms *in vivo*, and higher-order oligomers (*12*, *13*).

Several studies on isolated subunits of human erythrocyte spectrin have been published, and the reported properties of the isolated subunits vary considerably (*11*, *14*−*18*). It was observed that isolated β-subunits were prone to aggregation (*14*). However, this has not been observed by other researchers (*16*, *17*). Isolated α-subunits have been observed to migrate as several indistinct bands on nondenaturing gels and, under the electron microscope, to form simple ropelike structures to complex aggregated ringlets (*16*). On the other hand, homo-oligomers were not seen with isolated α- or β-subunits in rotary-shadow images (*17*). In addition, Coleman *et al.* (*17*), on the basis of their morphometric analysis, suggested that the β-subunit dominantly contributes to the overall flexibility and linearity of spectrin heterodimers and tetramers; they also suggest that the association of the subunits to form the heterodimer does not appear to impart any additional rigidity on the complex (on a rigidity per mass basis). In contrast, a rapid HPLC gel filtration assay suggested that both α- and β-monomers must be more flexible than dimers (*11*) and therefore adopt more compact configurations.

The variation in the properties of the isolated spectrin subunits might have arisen from difficulties in the purification, which, in part, are due to the strong noncovalent association of the subunits. Researchers have traditionally dissociated spectrin using strong chaotropic reagents such as urea (*14*) or detergents such as SDS (*15*). But those conditions might have been harsh enough to denature the protein to the extent that total renaturation was no longer possible. The most commonly used method to isolate spectrin subunits is that introduced by Yoshino and Marchesi (*16*) in which 3 M urea is used to dissociate the subunits. However, Speicher *et al.* (*11*) showed that such preparations probably contain 10−20% of monomers which can no longer recombine to form native-like heterodimers.

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In this report, we introduce an alternative method for the purification of spectrin subunits in which the subunits are dissociated at alkaline pH (∼11) and are then separated by ion-exchange FPLC (at pH 11) followed by size-exclusion FPLC (at pH 7.5). The use of FPLC has made this method reproducible, rapid, and easy. Following the isolation, the physical properties of the isolated subunits and reconstituted heterodimers were studied by means of circular dichroism, nondenaturing gel electrophoresis, and analytical ultracentrifugation. Our results show that the secondary structure of the purified α- and β-subunits and the reconstituted heterodimer are virtually identical to that of native spectrin. They also show that the thermal stability of the α- and β-subunits is very similar to that of reconstituted heterodimer and native spectrin. Sedimentation equilibrium studies showed that neither the α- nor β-subunits self-associate, while the reconstituted heterodimer underwent normal polymerization to tetramer as judged by nondenaturing gel electrophoresis as well as by sedimentation equilibrium analysis. The sedimentation coefficients of the reconstituted dimers and tetramers were indistinguishable from the equivalent native spectrin oligomers.

MATERIALS AND METHODS

Preparation of Crude Spectrin Extract and Spectrin Heterodimer. The methodology for these preparations is outlined in the accompanying paper (*50*).

Purification of Spectrin α- and β-Subunits by FPLC. Purification of spectrin subunits was performed using an FPLC system (Pharmacia) at 20 °C. Concentrated crude spectrin extract in the amount of 0.1−0.15 mL was mixed with ∼10 vol of buffer A (20 mM piperazine, pH 11). The sample was then injected onto a Mono Q HR 5/5 column (1 mL; Pharmacia) which was pre-equilibrated with 0.4 M NaCl, 20 mM piperazine, pH 11 (i.e., 80% buffer A + 20% buffer B, where buffer B was comprised of 2 M NaCl, 20 mM piperazine, pH 11). Either a linear (20% B for 7 min, then 20−30% B over 30 min) or a stepwise (20% B for 7 min, then 23.5% B for 20 min, and then 30% B for 10 min) gradient was used to elute, respectively, contaminating proteins (e.g., actin, protein 4.1, hemoglobin), β-spectrin and α-spectrin. The column was then cleaned with 100% B for 5 min and re-equilibrated with 20% buffer B. The amount of protein loaded was kept below 5 mg/run to obtain the best resolution. The flow rate was 0.5 mL/min, and the fraction size was 0.5 mL. Fractions of α- and β-spectrin were collected immediately, pooled separately, and dialyzed against 1 L of buffer C (10 mM sodium phosphate, 100 mM NaCl, 5 mM EDTA, 0.1 mM DTT,[1] 0.3 mM PMSF, and 0.02% NaN_3, pH 7.5) overnight at 4 °C. The dialyzed α- and β-spectrin samples were then concentrated to 0.5−1 mL with Aquacide III (Calbiochem) and individually applied to a Superose 6 HR 10/30 column (24 mL; Pharmacia) which was pre-equilibrated with buffer C. The proteins were eluted at a rate of 0.25 mL/min. Loading volume was kept below 0.5 mL to obtain the best resolution. Fractions (0.5 mL/tube) of α- and β-subunits were pooled separately. The pooled samples were concentrated with Aquacide II (Calbiochem), when necessary, and used for further experimental work.

Reassociation of the Isolated Subunits. To reconstitute heterodimer from the isolated subunits, equal moles of α- and β-subunits were mixed in specified buffers on ice. These were (i) buffer C for nondenaturing gels, (ii) 100 mM NaF, 5 mM sodium phosphate, 0.1 mM DTT, pH 7.5, for circular dichroism, and (iii) 100 mM NaCl, 5 mM sodium phosphate, 0.1 mM DTT, pH 7.5, for sedimentation analysis. Concentrations of the subunits were determined from the absorbance at 280 nm using extinction coefficients ($E_{1g/L,1cm} = 1.10$ for α-subunit, $E_{1g/L,1cm} = 1.18$ for β-subunit) calculated from the amino acid composition (*7*, *8*) according to the method of Gill and von Hippel (*19*). To determine the concentrations of native spectrin and reconstituted heterodimer, an extinction coefficient of $E_{1g/L,1cm} = 1.14$ was used.

Polyacrylamide Gel Electrophoresis. SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (*20*). Concentrations of acrylamide for the stacking gel and the resolving gel were 2 and 6%, respectively. Nondenaturing polyacrylamide gel electrophoresis was performed on slab gels with a linear 2−10% (w/v) polyacrylamide gradient. The buffer was composed of 0.04 M Tris, 0.02 M sodium acetate, and 2 mM EDTA adjusted to pH 7.4 with acetic acid (modified method of Fairbanks *et al*. (*21*)). Samples of native spectrin dimer and the reconstituted αβ-dimer were incubated at 30 °C for 1 h before the electrophoresis to promote tetramer formation. The protein concentration of all samples loaded onto the gel was 1 g/L. The gels were pre-run for at least 1 h at 50 V at 4 °C to remove excess ammonium persulfate and then electrophoresed for 19 h at 50 V at 4 °C. The buffer was continuously recirculated during the run to minimize pH changes.

Circular Dichroism Measurements. CD spectra were collected using a Jasco J-720 spectropolarimeter. Samples were dialyzed against 100 mM NaF, 5 mM sodium phosphate, and 0.1 mM DTT, pH 7.5, at 4 °C overnight prior to the experiments. For wavelength scans between 260 and 180 nm, samples (0.1 g/L) were placed in a 1 mm pathlength cell and scanned at a speed of 20 nm/min with a response time of 1 s, a band width of 1.0 nm, and a resolution of 0.5 nm at 20 °C. Four acquisitions were co-added. Buffer spectra were obtained in the same way and were subtracted from the sample spectra. The raw data were converted to mean residue ellipticity, $[\theta]_{MRW}$, using a calculated mean residue weight of 115.2 (*7*, *8*). For temperature scans, samples (0.1 g/L) in the 1 mm cell were placed in a cell holder whose temperature was controlled by the Jasco software and a Neslab RTE-111 water bath. Mean residue ellipticity at 222 nm, $[\theta]_{222}$, was measured with a response time of 1 s, band width of 1.0 nm, and resolution of 0.2 °C. The same results were obtained with ramp speed of 0.5 and 1.0 °C/min. The difference in temperature between the cell and the water bath was calibrated with a thermocouple prior to the experiments, and correction was made when the data were analyzed.

The thermal unfolding of the samples was analyzed assuming a 2-state transition mechanism between the native

[1] Abbreviations: $[\theta]_{MRW}$, mean residue ellipticity; $[\theta]_{222}$, mean residue ellipticity at 222 nm; A_{280}, absorbance at 280 nm; A_{294}, absorbance at 294 nm; DTT, dithiothreitol; *f*, frictional coefficient; PMSF, phenylmethanesulfonyl fluoride; $s_{20,w}$, sedimentation coefficient corrected to water at 20 °C; $s^0_{20,w}$, $s_{20,w}$ extrapolated to zero solute concentration; PAGE, polyacrylamide gel electrophoresis.

state and unfolded state. The plots of $[\theta]_{222}$ vs temperature were fitted using nonlinear regression with the following equation:

$$\theta_t = \theta_0 + at + [(\theta_\infty + bt) - (\theta_0 + at)] \exp[d(t - t_m)]/\{1 + \exp[d(t - t_m)]\} \quad (1)$$

where θ_0 and θ_∞ are the starting and ending ellipticity, respectively, a and b are the slopes of the linear part of the temperature dependence curves at the beginning and at the end, respectively, t_m is the melting temperature, d is the dispersion of the data, and t is the temperature at a given ellipticity, θ_t. The regression analysis returned values for each of these parameters ± standard error.

Analytical Ultracentrifugation. Sedimentation velocity and sedimentation equilibrium experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge. Protein samples were dialyzed against a buffer comprised of 5 mM sodium phosphate (pH 7.5), 100 mM NaCl, and 0.1 mM DTT at 4 °C overnight prior to the experiments.

In sedimentation velocity experiments, samples (400 μL) and buffer alone (420 μL) were placed in cells fitted with double-sector centerpieces and quartz windows. Native spectrin and reconstituted $\alpha\beta$-heterodimer were centrifuged at 42 000 rpm, and purified monomers were centrifuged at 48 000 rpm at 20 °C. Ten scans were taken at either 230 nm (when sample concentration was ≤0.2 g/L) or 280 nm (when sample concentration was >0.2 g/L) at intervals of 10 min. The scans at 230 and 280 nm were corrected for base-line deviation due to partial masking or window defects by subtracting the scans at 360 nm which were taken at 3000 rpm before the each high-speed run.

In sedimentation equilibrium experiments, three different concentrations (usually ∼1.0, 0.5, 0.25 g/L) of sample (120 μL) were placed parallel to the buffer against which the protein had been dialyzed (125 μL) in cells fitted with a 12 mm Yphantis 6-channel centerpiece. Samples of native spectrin and reconstituted $\alpha\beta$-heterodimer were centrifuged at 7000 rpm at 30 °C, and purified α- and β-subunits were centrifuged at 9300 rpm at 20 and 30 °C. After 48 h of centrifugation, scans were taken at 280 and 360 nm at intervals of 4−8 h. Sedimentation equilibrium was judged to have been reached when the difference in concentration distribution between two consecutive scans was zero. The correction for the base-line deviation due to partial masking or window defects was made by subtracting the scans at 360 nm from the scans at 280 or 230 nm.

Analysis of the Data from Sedimentation Experiments. Data analysis programs, XLAVEL and VELGAMMA (Beckman), were used to calculate the sedimentation coefficients and the diffusion coefficients from the sedimentation velocity experiments. In the program XLAVEL, the inflection points in the raw data of optical density (OD) vs radius were determined from the maxima of the first-derivative curves. The following equation was used for the calculation of the sedimentation coefficient, s:

$$s = \ln(r_{infl}/r_0)/\omega^2 t \quad (2)$$

where r_{infl} and r_0 are the radial positions of the inflection point and the meniscus, respectively, of the sedimentation profile, ω is the angular velocity of the centrifuge, and t is the elapsed time.

The weight-average sedimentation coefficients, s_{av}, were calculated with VELGAMMA, using the second moment analysis of Goldberg (*22*) to locate the boundary position ($r_{centroid}$). Values of s_{av} are calculated from the slope of plots of $\ln(r_{centroid})$ vs $\omega^2 t$ using the following equation:

$$s_{av} = \ln(r_{centroid}/r_0)/\omega^2 t \quad (3)$$

In addition, the diffusion coefficients were determined using VELGAMMA based on the algorithm described by Muramatsu and Minton (*23*). For each scan, a function $Z(t)$, which is based on a fractional concentration change across the boundary, is determined:

$$Z(t) = (\pi D/4)t + C \quad (4)$$

where D is the diffusion coefficient, t is the elapsed time, and C is a constant. Values of D are calculated from the slope of plots of $Z(t)$ vs t.

Extrapolation of plots of s (or s_{av}) vs concentration to zero concentration in order to obtain values of s^0 were guided by the following equation:

$$s \approx s^0(1 - k_s c_0) \quad (5)$$

where c_0 is the initial loading concentration of the protein in the grams per liter scale and k_s is a measure of the concentration dependence. Then the values of s^0 were corrected to water at 20 °C using the following equation:

$$s^0_{20,w} = s^0[(1 - \bar{\nu}\rho)_{20,w}\eta_{T,b}]/[(1 - \bar{\nu}\rho)_{T,b}\eta_{20,w}] \quad (6)$$

where $\bar{\nu}$ is the partial specific volume of the protein, ρ is the density of the solvent, η is the viscosity of the solvent, and b and T refer to the buffer composition and the absolute temperature of the experiment, respectively (*24*).

The sedimentation equilibrium data for isolated spectrin subunits were analyzed using the nonlinear regression program NONLIN (*25*, *26*). The optical density (OD) vs radius data for three different loading concentrations were analyzed simultaneously and were fitted directly with a single nonideal species model. This program returns both upper and lower values for each variable parameter, reflecting the asymmetrical distribution of the error space.

For native spectrin and reconstituted $\alpha\beta$-dimer, apparent weight-average molecular weights ($M_{w,app}$) were calculated from ln $c(r)$ vs radial position (r) data (*27*) obtained from sedimentation equilibrum experiments:

$$M_{w,app}(r) = 1/\phi_1[\mathrm{d} \ln c(r)/\mathrm{d}r^2] \quad (7)$$

where $\phi_1 = (1 - \bar{\nu}\rho)\omega^2/2RT$, R is the universal gas constant, and $c(r)$ is the total protein concentration at radial position r. A value 0.733 mL/g was used for $\bar{\nu}$ (*28*), and ρ was calculated to be 1.002 g/mL (*29*).

The concentration vs radius data were also used to calculate omega function values, $\Omega(r)$ (*30*):

$$\Omega(r) = c(r)\exp[\phi_1 M_1(r_F^2 - r^2)]/c(r_F) \quad (8)$$

$$\Omega(r) = a_1(r_F)c(r)/a_1(r)c(r_F) \quad (9)$$

where M_1 is the molar mass of the protomer, $c(r_F)$ is the total protein concentrations at reference radial position r_F, and a_1 is the thermodynamic activity of the protomer. For the analysis of data obtained for native spectrin, M_1 was taken as 526 000 g/mol (*7*, *8*), while for experiments with reconstituted $\alpha\beta$-dimer, M_1 was taken as 263 000 g/mol. A $c(r_F)$ of 0.2 g/L was chosen, which was common to all channels analyzed. To test for sample homogeneity and sedimentation equilibrium, the $\Omega(r)$ vs $c(r)$ curves were examined for coincidence over the common concentration range (*30*).

The SEK I self-association model (*31*, *32*) was used to fit both the apparent point-average weight-average molecular weight and omega function data for native spectrin. For this indefinite self-association model, a single equilibrium constant, K_{iso}, describes the sequential addition of heterodimers and the second virial coefficient, B, is a measure of nonideality. The SEK IV self-association model (*31*, *33*) was used to fit the apparent point-average weight-average molecular weight and omega function data for the reconstituted $\alpha\beta$-dimer. For this model, a separate equilibrium constant, K_{12}, describes the formation of dimer from α- and β-subunits, and K_{iso} again describes the sequential addition of heterodimers.

RESULTS

Isolation of Spectrin Subunits at Alkaline pH. As reported in the accompanying paper (*50*), we found that spectrin oligomers dissociate almost completely into individual α- and β-subunits at ≥pH 10.5. We exploited this finding to develop a method for the purification of α- and β-spectrin subunits using ion-exchange FPLC at pH 11 followed by size-exclusion FPLC at physiological pH. Spectrin extract (containing primarily spectrin and actin) was loaded onto a Mono Q ion-exchange column and fractionated at pH 11 using either a linear or a stepwise salt gradient at 20 °C. In general, a stepwise gradient gave better separation of the α-subunit peak from the β-subunit peak. Figure 1 shows a typical elution profile obtained by a stepwise salt gradient. At 0.4 M NaCl, contaminating proteins (e.g., actin and hemoglobin) were eluted, then the β-subunit was eluted at 0.47 M NaCl prior to the α-subunit which was eluted at 0.6 M NaCl. The fractions of α- and β-subunits were then dialyzed in buffer C (pH 7.5) to lower the pH. During the dialysis, the presence of DTT in the buffer was important to prevent aggregation of the proteins. The α-subunit fractions usually contained some β-subunit (0−10%), as determined by SDS−PAGE (data not shown). This β-subunit was in the form of heterodimers which were intractable to dissociation [see Figure 1 in the accompanying paper (*50*)]. For this reason, the α-subunit fractions were further purified on a Superose 6 size-exclusion column at pH 7.5 to remove residual spectrin heterodimer. The β-subunit fractions occasionally contained free α-subunits, and for this reason pooled β-subunit fractions were regularly subjected to Superose 6 chromatography.

Polyacrylamide Gel Electrophoresis. Following ion-exchange and size-exclusion chromatography, the isolated spectrin subunits were electrophoresed on SDS polyacrylamide and nondenaturing gels to examine their purities and their association properties (Figure 2). On nondenaturing

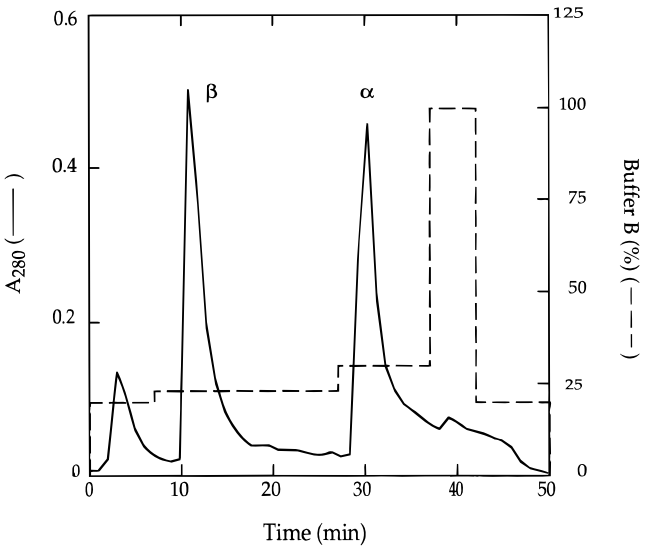

FIGURE 1: Isolation of spectrin subunits using ion-exchange FPLC. Spectrin extract dissolved in buffer A (20 mM piperazine, pH 11) was applied to a Mono Q HR 5/5 (Pharmacia) pre-equilibrated with 20% buffer B (20 mM piperazine, 2 M NaCl, pH 11). β-subunits and α-subunits were eluted in the second and the third peaks, respectively, using a stepwise salt gradient (23.5% B for 20 min, 30% B for 10 min). Minor proteins (i.e., actin, band 4.1, hemoglobin, etc.) were eluted at 20% B (first peak).

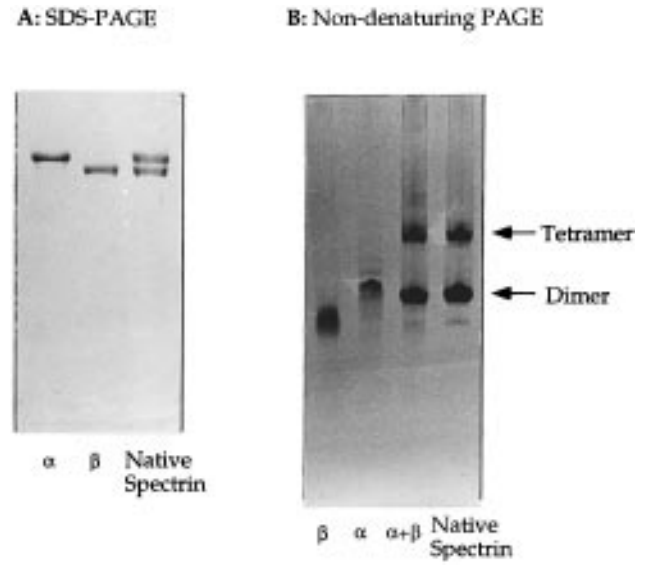

FIGURE 2: Polyacrylamide gel electrophoresis of α- and β-subunits of spectrin, reconstituted $\alpha\beta$-heterodimers, and native spectrin. (A) SDS−PAGE (6% polyacrylamide). (B) Nondenaturing PAGE (2−10% polyacrylamide gradient). Sample concentrations were 1 g/L. The samples of α- and β-subunits were purified by a combination of anion-exchange and size-exclusion chromatography. Samples of native spectrin and the reconstituted $\alpha\beta$-heterodimers ($\alpha + \beta$) were incubated at 30 °C for 1 h in buffer C prior to electrophoresis to promote self-association. The recombined α- and β-subunits were able to assemble into heterodimers and further self-associate into tetramers in a manner similar to that of native spectrin.

gels, the β-subunit band had broadness and mobility similar to that seen by Yoshino and Marchesi (*16*). For the α-subunit, several bands were observed, but the mobility and relative intensity of these bands were very different from those observed by Yoshino and Marchesi (*16*).

The isolated α- and β-subunits mixed at equimolar concentrations in buffer C retained the ability to assemble into heterodimers and tetramers (Figure 2B). Once the α-

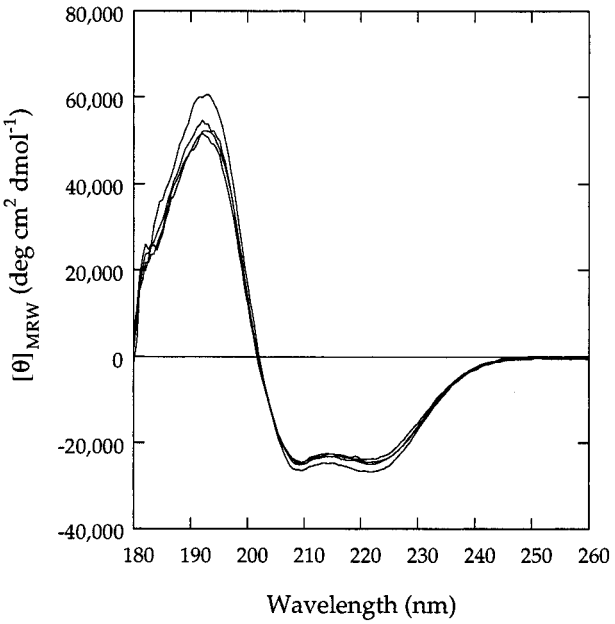

FIGURE 3: CD spectra of α- and *β*-spectrin, reconstituted α*β*-heterodimer, and native spectrin heterodimer. Sample concentrations were 0.1 g/L in a buffer consisting of 100 mM NaF, 5 mM sodium phosphate (pH 7.5), and 0.1 mM DTT. Samples were placed in a 1 mm pathlength cell, and four wavelength scans were collected and co-added. The raw data were converted to mean residue ellipticity, $[\theta]_{MRW}$, using a mean residue weight of 115.2. At the minimum at 222 nm and the maximum at 195 nm, the order of the spectra with respect to $[\theta]_{MRW}$, from greatest to least magnitude, is native spectrin, reconstituted α*β*-heterodimer, *β*-subunit, and α-subunit.

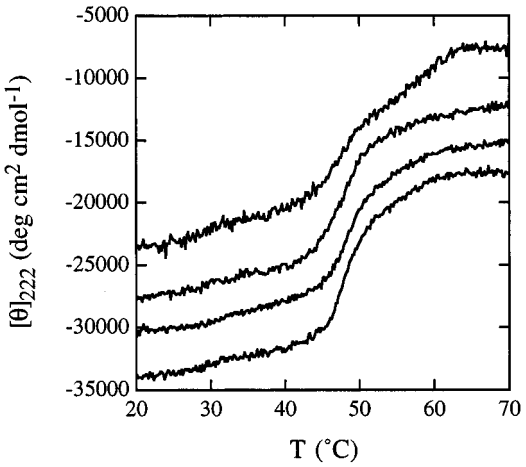

FIGURE 4: Temperature dependence of the mean residue ellipticity at 222 nm, $[\theta]_{222}$, of α- and *β*-subunits, reconstituted α*β*-heterodimer, and native spectrin. The data were fitted with eq 1 to obtain the melting temperature, t_m. The returned values of t_m for the α-subunit, *β*-subunit, reconstituted α*β*-heterodimer, and native spectrin were 46.6 (±0.3), 47.5 (±0.1), 48.0 (±0.1), and 47.6 (±0.1) °C, respectively. The order of spectra from top to bottom is α-subunit, *β*-subunit, reconstituted α*β*-heterodimer, and native spectrin where, for clarity, the $[\theta]_{222}$ values for the spectra have had 0, −3000, −6000, and −9000 deg cm^2 dmol^{-1} added, respectively.

and *β*-subunits were recombined, the resulting dimer and tetramer bands in nondenaturing gels were as sharp as those of native spectrin. Consistent with the work of Speicher *et al*. (*11*), the isolated α- and *β*-subunits readily reassociated to form heterodimers, even at low temperatures (0−4 °C), and did not require dialysis under denaturing conditions (*15*, *34*) to promote reassociation. In addition, mixing the isolated α- and *β*-subunits at low temperatures (0−4 °C) produced not only heterodimers but also tetramers (data not shown), consistent with previous work (*35*). Tetramerization was enhanced by 1 h incubation at 30 °C prior to electrophoresis, and it was further enhanced by the presence of glycerol (up to 60% (v/v)) during the 30 °C incubation (data not shown).

*CD Spectra of the Isolated Subunits and Recombined α*β*-Heterodimer*. As shown in Figure 3, the CD spectra of the isolated α- and *β*-subunits reflect only a very small loss of α-helix (∼5%) compared with that of native spectrin, as measured by the value of $[\theta]_{MRW}$ at 222 nm. The difference may be real, but it is within the error expected for spectrophotometric determination of protein concentraton and for the estimated values of the extinction coefficients. When the subunits were recombined to form the α*β*-heterodimer, the CD spectrum was also closely similar to that for native spectrin.

*Thermal Stability of the Isolated Subunits and Reconstituted α*β*-Heterodimer*. To examine the thermal stability of the isolated subunits and reconstituted α*β*-heterodimer, the ellipticity at 222 nm was used as a measure of α-helix content as the temperature was raised from 20 to 70 °C (Figure 4). The temperature dependence of the ellipticity of native spectrin showed a cooperative conformational transition centered at 47.6 (±0.1) °C. There appears also to be a less well-defined second transition at ∼55 °C. These features of native spectrin thermal denaturation are consistent with published results (*36*). The isolated *β*-subunit displayed a cooperative one-step transition centered at 47.5 (±0.1) °C. The temperature dependence of the isolated α-subunit had a more gradual transition centered at 46.6 (±0.3) °C, and there appears to be a second transition centered at ∼55 °C similar to native spectrin. These data indicate that the isolated α-subunits are thermally slightly less stable than the native dimers or *β*-subunits. When the two subunits were recombined, the temperature dependence was closely similar to that of native spectrin with a melting temperature 48.0 (±0.1) °C, suggesting that the thermal stability of spectrin is enhanced slightly by the association of the two component subunits.

*Concentration Dependence of the Sedimentation Coefficients of Isolated Subunits and Reconstituted α*β*-Heterodimer*. Sedimentation coefficients of the isolated α- and *β*-subunits were measured as a function of protein concentration by sedimentation velocity. Figure 5A shows a set of data for the concentration dependence of the sedimentation coefficients ($s_{20,w}$) for α- and *β*-subunits. On average, the $s_{20,w}$ values of the α- and *β*-subunits both increased slightly, being more apparent with the *β*-subunit, as the concentration increased from 0.05−1.0 g/L. The values of the coefficients extrapolated to zero concentration ($s^0_{20,w}$) were consistent through all preparations examined despite apparent variation in the slope of the lines of the concentration dependence. The slight increase in $s_{20,w}$ (especially for the *β*-subunit) as a function of increasing concentration suggests that very weak homo-associations occurred. The $s^0_{20,w}$ values were 6.28 and 5.74 S for the α- and the *β*-subunits, respectively. When the two subunits were recombined, the sedimentation coefficients for α*β*-heterodimer and -tetramer displayed concentration dependences indistinguishable from those of native spectrin dimer and tetramer (Figure 5B). The $s^0_{20,w}$

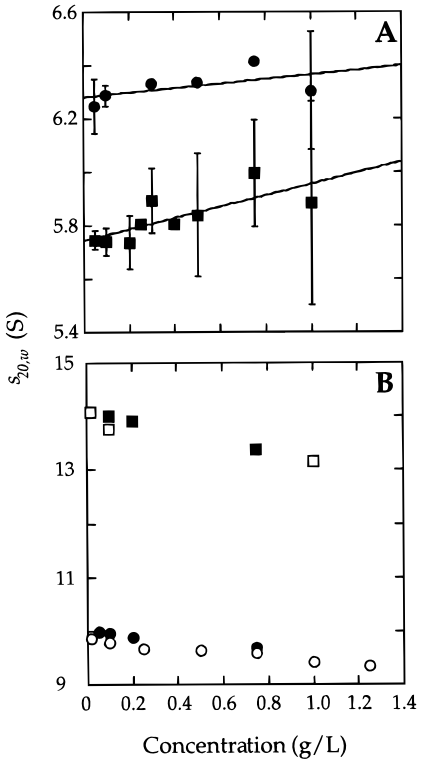

FIGURE 5: Concentration dependence of the sedimentation coefficients of the isolated spectrin α-subunit, β-subunit, reconstituted $\alpha\beta$-heterodimer and -tetramer, and native spectrin. (A) Plot of $s_{20,w}$ vs initial loading concentration of protein for the isolated α-subunit (●) and β-subunit (■). The solid lines represent linear regression fits to the data. The returned values of the slope are 0.084 (±0.062) for the α-subunit and 0.211 (±0.068) for the β-subunit where the errors represent the standard deviations of the fits. The error bars indicate the standard deviations of the means for 3−6 data points. (B) $s_{20,w}$ values for reconstituted $\alpha\beta$-heterodimer (●) and -tetramer (■) are compared to those of native dimers (○) and tetramers (□).

values for the heterodimer and tetramer were 9.7 and 13.7 S, respectively. The frictional coefficients (f) of the proteins calculated from the $s^0_{20,w}$ values were 2.0×10^{-7}, 1.9×10^{-7}, and 2.43×10^{-7} g s^{-1} for the α-subunit, β-subunit, and native spectrin dimer, respectively. The ratios of f to f_0 (the calculated frictional coefficient for the protein if it were an unhydrated, incompressible sphere of the equivalent molecular weight and partial specific volume) were 2.42, 2.45, and 2.41 for the α-subunit, β-subunit, and native spectrin dimer, respectively.

Sedimentation Equilibrium Experiments. Sedimentation equilibrium experiments were carried out on the isolated spectrin subunits and reconstituted $\alpha\beta$-heterodimer in order to investigate their association behavior. Figure 6A shows a nonlinear regression fit of the concentration distribution of the isolated α-subunit. The concentration distribution was fitted well with a single nonideal species model, and the returned value of the molecular mass was 277 (−45, +44) kDa while that of the second virial coefficient was 2.18 (±1.26) × 10^{-6} L mol g^{-2}. The concentration distribution of the isolated β-subunit (not shown) was also fitted well with a single nonideal species model, and the returned value of molecular weight was 250 (−45, +45) kDa while that of the second virial coefficient was 1.42 (±1.72) × 10^{-6} L mol g^{-2}.

Figure 7 shows plots of apparent molecular weight vs $c(r)$ for native spectrin and reconstituted $\alpha\beta$-dimer. Fitting the

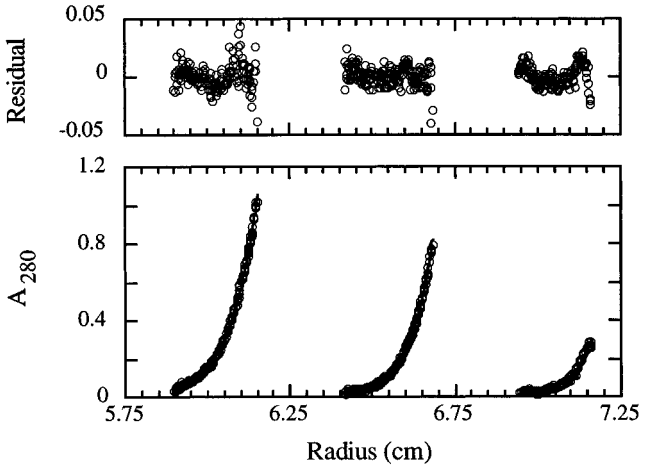

FIGURE 6: Analysis of sedimentation equilibrium data obtained for the isolated α-subunit. Three initial loading concentrations of protein (1.0, 0.5, 0.25 g/L) were loaded into a Yphantis 6-channel centerpiece and centrifuged to sedimentation equilibrium at 9300 rpm at 20 °C. The concentration distribution was measured by recording the radial dependence of the absorbance at 280 nm and correcting for the radial dependence of the absorbance at 360 nm. The three plots of absorbance vs radius were then fitted simultaneously with a single nonideal species model using the nonlinear regression program NONLIN (Yphantis, 1964; Johnson *et al.*, 1981). The fits to the three channels of data are described by the solid lines, and the residuals to the fit are shown in the top panel. The returned values of the molecular weight and the nonideality parameter, B, were 277 (−45, +44) kDa and 2.18 (±1.26) × 10^{-6} L mol g^{-2}, respectively.

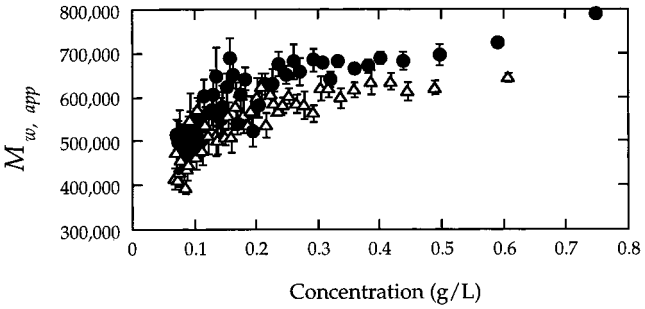

FIGURE 7: Apparent weight-average molecular weight distribution for the reconstituted $\alpha\beta$-heterodimer (△) and native spectrin (●). Three initial loading concentrations of native spectrin (1.0, 0.5, 0.25 g/L) and reconstituted $\alpha\beta$-heterodimer (0.8, 0.4, 0.2 g/L) were loaded into a Yphantis 6-channel centerpiece and centrifuged to sedimentation equilibrium at 7000 rpm at 30 °C. Apparent weight-average molecular weights ($M_{w,app}$) were calculated from ln $c(r)$ vs radial position (r) data using eq 7, where $c(r)$ is the total protein concentration at radial position r. The $M_{w,app}$ values were averaged six points at a time, and the error bars represent the standard errors of the means.

native spectrin data with the SEK I model (in which heterodimers can be added sequentially with the same change in free energy each time) returned a value of $K_{iso} = 1.87$ (±0.26) × 10^6 M^{-1}. This value is consistent with published values (*13*). The value of K_{iso} for the reconstituted spectrin was smaller than that of native spectrin, probably due to an effect of contaminating monomers which are not participating in polymerization. When a plot of preaveraged $\Omega(r)$ vs $c(r)$ for the reconstituted $\alpha\beta$-dimer was fitted with the SEK IV model, in which the protomers of the self-association are monomers ($M_1 = 263\,000$), we obtained values of $K_{12} = 12$ (±1.9) × 10^6 M^{-1} and $K_{iso} = 4.5$ (±0.63) × 10^6 M^{-1}. The results indicate there is a small proportion (<10% at a total

spectrin concentration of 1.0 g/L) of monomers that are not polymerized into oligomers. The existence of these unpolymerized monomers may be due to a mismatch in molar concentration between α- and β-spectrin.

DISCUSSION

The Purification Protocol. We have developed a new method for the purification of spectrin subunits which provides α- and β-subunits (i) which have high purity (Figures 1, 2), (ii) which contain levels of secondary structure and thermal stability closely similar to those of native spectrin (Figures 3, 4), and (iii) which possess the ability to recombine to form dimers and tetramers with hydrodynamic and self-association properties closely similar to those of native spectrin (Figures 5, 7).

The method requires the use of alkaline pH as high as 11. We showed that this pH is sufficient to dissociate almost all of the spectrin dimer and higher oligomers to the component monomer subunits [see Figures 1, 2 in the accompanying paper (*50*)]. The dissociation at high pH is accompanied by some unfolding of the spectrin molecule [see Figure 2B in the accompanying paper (*50*)] but this is reversible in the short term if the pH is kept at ≤11.

The purification requires the use of a Mono Q anion-exchange column which is stable at pH 11. The combination of Mono Q with an FPLC system made this purification fast, easy, and reproducible. The following size-exclusion FPLC was chiefly required because the α-subunit peak was partly contaminated by undissociated dimer. This undissociated dimer remains refractory to dissociation even up to pH 13 [see Figure 1 in the accompanying paper (*50*)]. Observations of a similar subpopulation of spectrin dimers refractory to dissociation have been reported for the isolation of spectrin subunits in the presence of urea (*34*, *37*). Pedroni *et al*. (*37*) pointed out a possible link between undissociated spectrin at urea concentrations up to 3.5 M and the phosphorylation state of the β-subunit. The β-subunits in the undissociated dimers were more highly phosphorylated than the β-subunits derived from the dissociable dimers. In addition, the β-subunit was also subjected to size-exclusion chromatography to reduce the presence of any unfolded protein and to remove occasional contamination by α-subunit.

The most commonly used method for preparing purified α- and β-subunits of spectrin is with the use of urea and a single ion-exchange chromatography step (*16*). However, Speicher *et al*. (*11*) showed that this method resulted in a substantial proportion of the subunits being apparently denatured and incapable of recombining to form αβ-dimers. Significant improvement was obtained by Speicher *et al*. by adding a size-exclusion chromatography step. Our method retains distinct advantages, however. First, the rapid equilibrium that appears to occur between monomer subunits and heterodimers in the presence of 3 M urea does not appear to occur at pH 11 [see Table 2 in the accompanying paper (*50*)]. This equilibrium may reduce the yield of monomers. Second, our method avoids the need for lengthy dialysis required to remove urea. There is also the potential to examine the properties of the dissociation-resistant dimers which can be purified using our method. In addition, we have undertaken a much more extensive series of experiments designed to assess the quality of the purified subunits and their biophysical properties than have other researchers.

Properties of the Purified α- and β-Subunits. The biophysical properties of the purified α- and β-subunits are very similar to the original spectrin polymers from which they were derived. The melting curves of the α- and β-subunits were sharp (t_m = 46.6 and 47.5 °C, respectively) and indicate that the α-subunit is only slightly less stable than the β-subunit. Furthermore, the values are very similar to that obtained for native dimer (47.6 °C; Figure 4). There is a second, broader transition in the α-subunit centered at ∼55 °C which is also observed in native dimer (*36*; Figure 4), suggesting that this is bestowed by the properties of the α-subunit.

The CD spectra for the isolated α- and β-subunits reflect only a very small loss of α-helix (∼5%) compared to that of native spectrin (as measured by the value of $[\theta]_{222}$). The difference may be real but is within the error expected for spectrophotometric determination of protein concentration and for the estimated values of the extinction coefficients. The small differences may also be due to the fact that we have removed a dissociation-resistant subpopulation of dimers.

There was a noticeable difference in the migration pattern of the purified α- and β-subunits in nondenaturing gels. The β-subunit displays a single band, while the α-subunit shows one main band and several minor bands (Figure 2B). Yoshino and Marchesi (*16*) also observed bands for the α-subunit which migrated to the positions expected of dimers and tetramers and interpreted this as self-association. This was consistent with their electron microscope data. The β-subunit adopted wormlike conformations and was almost exclusively monomeric, while the α-subunit varied from simple ropelike structures to complex aggregated ringlets (*16*). Other workers, however, have observed aggregation of the β-subunit using some purification protocols (*14*, *38*, *39*). Our sedimentation equilibrium data show that both subunits are largely monomeric up to a concentration of ∼1 g/L (Figure 6). There is no clear evidence from the concentration dependence of the sedimentation coefficient of the α-subunit that self-association occurs (Figure 5). However, the concentration dependence of the sedimentation coefficient of the β-subunit has a significant positive slope indicating that a very weak self-association occurs. The broad band for the α-subunit seen in our nondenaturing gels may represent a series of conformations which do not rapidly interconvert at the low temperatures used during electrophoresis.

Calvert *et al*. (*14*) reported sedimentation coefficient values of 5.5 S and an unspecified value of below 7 S for the isolated α- and β-subunits, respectively. However, their subunits were fractionated in 7 M urea, they required an intermediate dialysis in 6 M guanidine hydrochloride to improve renaturation, and the yield of dimer was only 50−70%. Cohen & Langley (*34*) obtained values of 6.55 and 7.05 S for α- and β-subunits, respectively, fractionated in 3 M urea. These data were obtained from sucrose gradient centrifugation where a set of proteins with known sedimentation coefficients are required for calibration. Our values for the sedimentation coefficients ($s^0_{20,w}$ values of 6.28 and 5.74 S for the isolated α- and β-subunits, respectively) have been obtained using sedimentation velocity in the analytical ultracentrifuge where sucrose gradients and a set of calibration markers are not required.

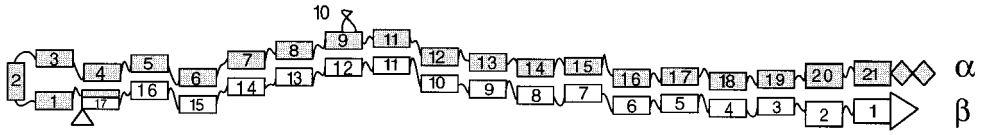

FIGURE 8: Model of the lateral association of the α- and *β*-chains of erythrocyte spectrin. The triple-helical repeat domains are represented by rectangles numbered from the N-terminal end of each chain. The flexible connecting regions between the repeats are represented by squiggles. Note that the flexible connectors and the folded repeat domains are "in register" along the length of the α- and *β*-chains of the dimer, in keeping with the sedimentation results showing that the wormlike flexibility of the dimer is very similar to that of the purified α- and *β*-chains. The "nucleation" repeats, which are involved in high-affinity interactions between the two chains, are represented by larger rectangles. Following binding at the nucleation repeats, the chains are envisaged to zip up via a series of lower affinity, and as yet very poorly understood, interactions along the lengths of the chains (*42*). The 17th repeat of the *β*-chain is a partial repeat containing only the first two helices of a normal bundle, while the N-terminus of the α-chain contains a partial repeat containing only the third helix of a normal bundle (*49*). These partial repeats interact to form a complete repeat (*49*) and thus the "closed" form of the dimer shown here. The closed form is consistent with electron micrographs of spectrin (*9*) and the thermodynamics and kinetics of the self-association process (*13*). The α-chain contains an SH-3-like domain (repeat 10; represented by the loop) and two EF-hand domains (diamonds) while the *β*-chain contains an actin-binding domain (large triangle) and phosphorylatable domain (small triangle), none of which are homologous to the repeat units.

Note that, with our method of purification and the use of analytical sedimentation, the sedimentation coefficient of the *β*-subunit is smaller than that of the α-subunit. This results in the frictional ratios (f/f_0) calculated from our values of $s^0_{20,w}$ being almost identical ($\alpha = 2.45$, $\beta = 2.42$), strongly indicating that both of these wormlike subunits can adopt a similar range of shapes in solution and that their flexibility is closely similar. This result is consistent with the gel-filtration data obtained by Speicher *et al*. (*11*) showing that the elution positions of the α- and *β*-subunits were, within error, the same, indicating no gross difference in hydrodynamic shape. The results, however, appear to be different from those obtained by Coleman *et al*. (*17*), who calculated average correlation lengths of purified α- and *β*-subunits from electron micrographs. The correlation length of the *β*-subunit (32 nm) was ~10× that of the α-subunit, indicating that the *β*-subunit is considerably less flexible. If this were the case, we would expect the frictional ratio of the *β*-subunit to substantially exceed that of the α-subunit. For example, that of the stiffer more rodlike brain spectrin tetramer ($f/f_0 = 3.3$) substantially exceeds that of the more flexible erythroid spectrin tetramer ($f/f_0 = 2.7$; *40*). It is possible that the results obtained by Coleman *et al*. (*17*) are artifactual due to the purification of the subunits using urea, the preparation of protein samples for electron microscopy, and the "flattening" of protein into two dimensions on the grids.

The value of f/f_0 for the dimer of spectrin was calculated to be 2.41. The value of f_0 increases as the $\sqrt[3]{M}$, where M is the molar mass of the molecule. Thus, f_0 for the dimer of spectrin will be ~25% larger than for the α- or *β*-subunits and, in order for the frictional ratio of the dimer to equal those of the α- and *β*-subunits, f must also be ~25% larger. Given that all three species have approximately equal contour lengths (~100 nm), a 25% increase in f for the dimer may result from an increased asymmetry due to the dimer being stiffer than the individual subunits. However, other factors will contribute to the increase in f for the dimer. These include the possibility of "draining" of solvent between the subunits as the dimer sediments. Our conclusion is that the flexibility of the dimer is somewhat, but not grossly, reduced compared to that of the individual subunits.

The physical basis of the wormlike bending of erythrocyte spectrin remains controversial with models for the source of the bending highlighting (i) the role of configurational entropy (*6*) associated with random-coil connecting regions between repeats (*41*, *42*), (ii) reversible hydrophobic contacts between repeats (*43*), and (iii) a combination of configurational entropy and the reversible unfolding of secondary structure adjacent to the unfolded connecting regions (*46*). It seems clear that bending does not occur *within* the folded triple-helical repeats (*42*), but that some flexible connector *between* repeats is required to allow the relative movement of one repeat with another. Our data show that the flexible connectors between repeats (and any other factor that may bear on the wormlike bending of spectrin such as reversible unfolding or binding between repeats) are essentially identical in the α- and *β*-subunits despite the sequence differences between the subunits. Furthermore, the very modest loss of flexibility of the spectrin dimer compared to that of the individual chains indicates that the flexible connectors in the α- and *β*-subunits are "in register" along the length of the spectrin dimer (Figure 8). If the flexible connectors were out of register, or staggered, between the α- and *β*-subunits, the dimer would probably be much stiffer than the individual subunits. A consequence of in-register flexible connectors is that the folded repeat domains would also be in register. That is, in the dimer, each folded α-chain repeat would pair with a single folded *β*-chain repeat beginning at the so-called nucleation site near the actin-binding end of the dimer and proceeding in a zipper-like fashion to the other end (*42*).

Reconstituted αβ Spectrin. The mixing of purified α- and *β*-subunits at equimolar concentrations resulted in the formation of dimers and tetramers essentially indistinguishable from native spectrin. Migration in nondenaturing gels (Figure 2B), secondary structure as measured by CD (Figure 3), thermal stability (Figure 4), $s^0_{20,w}$ values, and the concentration dependence of the sedimentation coefficient (Figure 5) were all closely similar to those of native protein.

Sedimentation equilibrium experiments on the reconstituted protein showed that the equilibrium constant describing the sequential addition of heterodimers was very similar to that for native spectrin. This is a strong quantitative indicator of the quality of the purified subunits. Some free monomer, however, was also present. That is, the apparent weight-average molecular weight dropped below the molecular weight of the heterodimer (526 000) as the total concentration of protein approached zero (Figure 7). This may be due to the presence of a small amount of association-incompetent monomer. Alternatively, it may result from a small mismatch in the molar concentrations of the α- and *β*-subunits when they were mixed. Based on the values of the returned

equilibrium constants, the percentage of monomer is ~10% when the total protein concentration is 1 g/L.

When purified α- and β-subunits were mixed at equimolar concentrations at 0−4 °C, tetramers were formed in addition to heterodimers (not shown). This result is consistent with that of Yoshino and Marchesi (*35*). Native dimers, on the other hand, convert to tetramers at an extremely slow rate at these temperatures (*44*). The tetramerization binding sites on the α- and β-subunit of a dimer normally form an intradimer bond—the so-called hairpin loop or ring form of the dimer (*13*, *45*). Breaking the bond and allowing two heterodimers to interact to form a tetramer is associated with a very high energy of activation barrier. When the individual subunits are mixed at low temperatures, however, some tetramers can evidently form prior to ring-closure of dimers.

In nucleated erythrocyte precursors, the α-subunit of spectrin is synthesised at a 4-fold excess over the β-subunit, and the unassembled subunits are rapidly degraded compared with assembled polymers (*46*, *47*, *48*). Our results indicate there is little or no difference in many of the physicochemical properties of purified, folded α- and β-subunits compared to dimers and tetramers. Thus, the protease resistance of polymers *in vivo* may result, in part, from "masking" of protease-sensitive sites due to the close apposition of α- and β-subunits along their contour length.

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