Analysis of Human Red Cell Spectrin Tetramer (Head-to-Head) Assembly Using Complementary Univalent Peptides†

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ABSTRACT: The mass-driven assembly of spectrin dimers to form tetramers involves two equal head-to-head α–β associations and requires at least 30 °C for interconversion to occur readily. In this paper, the properties of tetramer formation were investigated using two complementary univalent peptides (the αI domain and β monomers). Since the αI domain lacks an essential nucleation site required for side-to-side (lateral) heterodimer assembly [Speicher et al. (1992) *J. Biol. Chem. 267*, 14775–14782], these two peptides can only assemble head-to-head at a single site. This head-to-head assembly readily occurs at lower temperatures, indicating the temperature barrier for dimer–tetramer interconversion is caused by a conformational constraint of the dimer. This constraint, a closed hairpin loop, is released when the laterally associated partner is removed. The univalent αI–β binding affinity at 37 °C (K_a = 1.4 × 10^5 M^{-1}) is similar to the dimer–tetramer association constant at the same temperature. As the temperature is decreased from 37 to 0 °C, the αI–β binding affinity increases about 32-fold. In contrast with head-to-head associations involving dimers, the second-order rate constants of two complementary univalent peptides (i.e., αI and β) are dramatically higher, and the estimated activation energy (about 50 kJ mol^{-1}) is about 5-fold lower. An open dimer conformation is an obligatory high-energy intermediate required for dimer–tetramer interconversion, and opening the dimer hairpin loop contributes about 190 kJ mol^{-1} to the activation energy for tetramer association. Since closed dimers probably contain an internal head-to-head α–β association, the closed $\rightleftarrows$ open dimer equilibrium would shift toward closed dimers at lower temperatures and would dramatically slow dimer–tetramer interconversion at low temperatures. The current model defines the relative contributions of each α–β association in dimer–tetramer interconversion, and it also allows estimations of the relative proportions of open and closed dimers under different experimental and physiological conditions.

Spectrin, a predominant structural component of the erythrocyte membrane skeleton, is comprised of an α subunit, M_r = 280K, and a β subunit, M_r = 246K. Most of both subunits consist of contiguous, homologous, 106-residue segments or motifs (Speicher et al., 1983; Speicher & Marchesi, 1984; Sahr et al., 1990,; Winklemann et al., 1990). The first stage of spectrin assembly involves antiparallel, side-to-side association of the α and β subunits to form a flexible, rodlike heterodimer with an apparent molecular length of 100 nm as determined by electron microscopy (Shotton et al., 1979). This heterodimer assembly requires a specific nucleation site located near the actin binding domain (Speicher et al., 1992). Spectrin dimers can further associate head-to-head to form 200-nm-long tetramers. This head-to-head assembly of tetramers involves association of the N-terminal region of the α subunit with the C-terminal region of the β subunit, although the precise regions involved in binding have not been determined. Higher order oligomers form at high concentrations in solution (Morrow & Marchesi, 1981), and to a limited extent in situ, although the predominant form of spectrin on the membrane appears to be tetramers (Byers & Branton, 1985; Liu et al., 1987; Vertessy & Steck, 1989). The critical role of the head-to-head tetramer binding site in maintaining the architecture of the red cell membrane is clearly demonstrated by numerous hereditary hemolytic anemia-related mutations that perturb tetramer formation (Marchesi et al., 1987; Coetzer et al., 1990; Garbarz et al., 1990).

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The moderate-affinity head-to-head association between α and β at the tetramerization site is probably dynamic in vivo, with facile interchange between different tetramer complexes. In solution, the time required to achieve equilibrium is strongly temperature-dependent; even at 37 °C, at least 20 min is required to reach equilibrium while several hours are required at 30 °C. At lower temperatures, 0–4 °C, nonequilibrium states can be maintained for days or weeks. This "kinetic trapping" at lower temperatures has been ascribed to a high activation energy presumably involving a conformational change (Ungewickell & Gratzer, 1978). A closed-loop form of the dimer was initially suggested by electron microscopy images (Shotton et al., 1979) and has been incorporated into most models describing tetramer formation (Morrow & Marchesi, 1981; Morris & Ralston, 1989; Ralston, 1991). A reversible opening of the heterodimer at the head-to-head association site has been proposed as a key intermediate species based on thermodynamic arguments (Morris & Ralston, 1989). The importance of an intradimer α–β association at the tetramer association site is further supported by an analysis of tetramer formation using mutant univalent dimers (Morris et al., 1989). This previous study showed that two univalent dimers with complementary defective α and β sites could readily form tetramers at low temperatures, although measurement of association constants and thermodynamic parameters was not practical using the methods employed in that study.

In this study, we describe the head-to-head binding properties of the αI domain with β monomers using a high-performance liquid chromatography (HPLC)[1] gel filtration assay. Since the αI domain lacks a side-to-side nucleation

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site which is located near the actin binding site (Speicher et al., 1992), these two univalent peptides can only assemble head-to-head at the tetramerization site. In contrast with several earlier studies which used single-site peptide association with normal dimers (Morrow et al., 1980; Morrow & Marchesi, 1981; Shahbakhti & Gratzer, 1986), the use of β monomers instead of dimers permits evaluation of the role of the side-to-side paired subunit in head-to-head association. The determination of reaction rate constants and thermodynamic parameters for the univalent association permits the refinement of a thermodynamic model for spectrin tetramer formation.

EXPERIMENTAL PROCEDURES

Spectrin Extraction and Purification. Crude spectrin was isolated from human whole blood within 24 h of collection, and spectrin monomers were purified as previously described (Speicher et al., 1992). Spectrin dimers and tetramers were purified by gel filtration. Briefly, crude spectrin was concentrated by precipitation with 50% ammonium sulfate. The ammonium sulfate pellet was resuspended in 20–30 mL of isotonic KCl buffer (10 mM Tris, 20 mM NaCl, 130 mM KCl, 1 mM 2-mercaptoethanol, and 30 μM PMSF, pH 7.4) and then dialyzed in the same buffer. Spectrin dimers and tetramers were purified by chromatography on a Sepharose CL-4B column (5 × 90 cm, Pharmacia) in isotonic KCl at 1 mL/min. Peak fractions were pooled and concentrated by vacuum dialysis using a Micro-ProDiCon concentrator.

Preparation of αI Domain Spectrin Peptides. Crude spectrin was dialyzed into 20 mM Tris, 1 mM β-mercaptoethanol, and 0.02% azide, pH 7.6. The dialyzed crude spectrin was proteolyzed with trypsin at 0 °C for 90 min [E:S ratio = 1:100 (w/w)]. The αI domain was purified on a monoclonal antibody column and eluted with a solution of 100 mM Tris, 300 mM NaCl, and 6 M urea, pH 7.0, as previously described (Speicher et al., 1982). Purified αI domain peptides, primarily an apparent 80-kDa peptide, were dialyzed into isotonic KCl buffer.

Analytical HPLC Assay. Spectrin tetramers, dimers, monomers, and peptides were separated and quantified using three large-pore polymer-based HPLC columns (TSK 5000 PWXL, 7.8 mm × 30 cm). The columns and injector were generally either maintained at 4 °C for analysis of samples incubated at low temperatures or maintained at room temperature for 23, 30, and 37 °C samples. Columns and samples were in isotonic KCl buffer, pH 7.4. A flow rate of 0.40 mL/min was used for all runs except 23 °C rate constant experiments where a flow rate of 0.80 mL/min was used.

Quantitation of Reaction Components. HPLC protein peaks were monitored using the absorbance at 280 nm and intrinsic tryptophan fluorescence (excitation 280 nm, emission filter 370 nm). Peaks were integrated on a data acquisition system (PE Nelson Analytical, version 5.1). Peak height and peak area response factors were calculated by injecting known amounts of dimer, monomer, and αI domain samples. Protein concentrations were determined by amino acid analysis. Molecular weights used for molarity calculations were 246 000 for the β monomer and 73 000 for the αI domain. It should be noted that αI domain migrates anomalously on SDS gels with an apparent molecular weight of 80 000 although the actual molecular weight is substantially smaller and 73 000 is a more reasonable estimate.

[1] Abbreviations: HPLC, high-performance liquid chromatography; PBS, phosphate-buffered solution; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS sodium dodecyl sulfate.

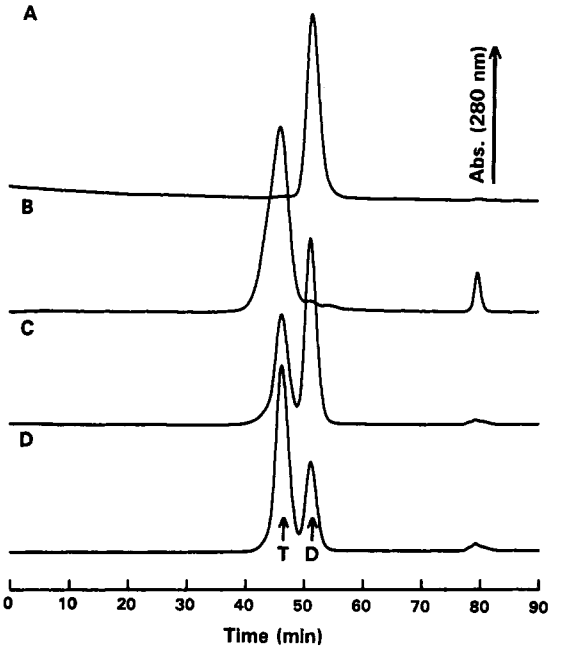

FIGURE 1: Separation of spectrin dimers and tetramers by HPLC gel filtration. Absorbance is in arbitrary units with the largest peak scaled to a uniform height in all chromatograms. (A and B) 80 μg from pools of the dimer and tetramer regions, respectively, from a Sepharose CL-4B separation maintained at 0 °C. (C) Dimer pool at 2.5 mg/mL incubated at 37 °C for 45 min immediately prior to injection. (D) Dimer pool at 2.5 mg/mL incubated at 30 °C for 2 h immediately prior to injection. Peak positions of tetramers and dimers are indicated by arrows.

Polyacrylamide Gel Electrophoresis. Dimers and tetramers after Sepharose CL-4B gel filtration were evaluated on native slab gels (3 × 100 mm) containing 4% acrylamide in 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH 7.4, as described by Morrow et al. (1980). Nondenaturing electrophoresis was performed at 4 °C with constant recirculation of electrode buffers. SDS–polyacrylamide gel electrophoresis was performed using 7% slab gels (1.5 × 100 mm) as described by Laemmli (1970).

RESULTS

Analysis of Dimer–Tetramer Equilibria Using an Analytical HPLC Gel Filtration Assay (2 Site + 2 Site). The HPLC gel filtration separation is a rapid and quantitative assay for spectrin head-to-head assembly using normal dimers (bivalent binding sites) (Figure 1) as well as smaller univalent peptides and monomers. Normal dimers and tetramers were initially analyzed to compare association constants of different numbers of sites on a homologous system as well as to compare with values previously determined using native gel electrophoresis (Morrow et al., 1980; Morrow & Marchesi, 1981; Shahbakhti & Gratzer, 1986) and ultracentrifugation (Ungewickell & Gratzer, 1978; Morris & Ralston, 1989). Elution positions were determined using dimer and tetramer pools from a Sepharose CL-4B separation (Figure 1A,B, respectively), and the identity of the peaks was confirmed by native gel electrophoresis. Base-line separation of dimers and tetramers could be obtained with elution times less than 60 min and total run times of 90 min, although hexamers were incompletely resolved as indicated by the leading shoulder on the tetramer peak in Figure 1B. At elevated temperatures (37 and 30°C), purified dimer fractions showed the expected mass-driven assembly into tetramers (Figure 1C,D), and association constants were in close agreement with those previously determined by other methods (Table I). Rechro-

Table I: Comparison of Association Constants (K_a) at 37 and 30 °C[a]

expt	no. of sites	temp (°C)	K_a (M^{-1})
HPLC assay: β–αI complex	1 + 1	37	1.4×10^5
HPLC assay: dimer–tetramer	2 + 2	37	1.8×10^5
Ungewickell and Gratzer (1978)	2 + 2	37	2×10^5
HPLC assay: β–αI complex	1 + 1	30	2.6×10^5
HPLC assay: dimer–tetramer	2 + 2	30	1.2×10^6
Ungewickell and Gratzer (1978)	2 + 2	29.5	1.5×10^6
Morrow et al. (1980) (dimer; 80K)	1 + 2	30	4×10^5
Eber et al. (1988) (truncated β)	1 + 2	30	5×10^5
Shahbakhti and Gratzer (1986)	2 + 2	30	1.1×10^6

[a] Association constants determined by the HPLC gel filtration method for one and two sites are compared with published values determined either by native gel electrophoresis or by ultracentrifuge sedimentation.

matography of dimer and tetramer fractions confirmed that little interconversion occurred below 30 °C (data not shown).

Association of β Monomers and the αI Domain with Dimers (1 Site + 2 Site). Head-to-head association of purified spectrin monomers with dimers showed similar binding properties to assembly of dimers alone. The univalent head-to-head monomer–dimer complexes migrated near the tetramer position as predicted from the expected molecular shape (double the original length or 200 nm). Most importantly, these complexes only formed above room temperature, similar to dimer–dimer assembly. Head-to-head complexes between dimers and the purified αI domain, which was previously shown to have an intact head-to-head binding site (Morrow et al., 1980), could also be formed at elevated temperatures and separated using the HPLC assay (data not shown). These experiments established that both two-site and single-site head-to-head complexes with dimers could be isolated by HPLC gel filtration. The expected temperature threshold for dimer head-to-head association was preserved using this technique.

Head-to-Head Association of β Monomers and the αI Domain at 0 °C (1 Site + 1 Site). The αI domain and β monomers were used to further evaluate the mechanism of head-to-head complex formation without any contribution from a paired (side-to-side) subunit. These two univalent components can only form a single head-to-head association since the αI domain does not contain the essential nucleation site for side-to-side association (Speicher et al., 1992).

As shown in Figure 2A, these peptides can readily associate at 0 °C in marked contrast to head-to-head assembly of dimers with either univalent peptides (αI domain or isolated subunits) or other dimers. By 24 h, essentially all of the limiting β monomer has been incorporated into the complex. SDS gel analysis of the bound fractions confirms the formation of αI–β complexes as shown in Figure 2B. The arrow highlights a minor proteolytic degradation product of the β monomer, missing about 10 kDa from the C-terminal, which cannot associate head-to-head with the αI domain. With the exception of this minor (<1%) proteolytic product, essentially all of the β monomers could form head-to-head complexes. Analogous experiments confirmed that essentially all of the αI domain peptide (>98%) could form head-to-head complexes (data not shown). Densitometric scans of the complex peak fractions were used to quantify the amounts of β monomers and αI domain in the complex peaks. A β:αI molar ratio of 1.04 ± 0.05 was obtained based on eight determinations. No more than one αI peptide could bind per β monomer even when a large molar excess of αI domain peptides was incubated with β monomers. Figure 2C shows that the rate of complex formation is concentration-dependent. At least 24 h is required to reach equilibrium at 1.15 mg/mL, while about 48 h is required at 0.21 mg/mL. Lower protein concentrations also permit an estimation of the forward rate constants.

Determination of Forward Rate Constants for Single-Site Head-to-Head Association. The association of the αI domain and β monomers follows second-order kinetics as shown in Figures 3 and 4. The association can be described by

$$\alpha\mathrm{I} + \beta \underset{k_-}{\overset{k_+}{\rightleftharpoons}} \alpha\mathrm{I}\beta$$

where k_+ is the forward rate constant and k_- is the rate constant for the reverse reaction. The kinetics of association at 0 °C using equimolar amounts of αI and β monomers are shown in Figure 3. Since the two reacting species are initially equimolar and are consumed at an equal rate, the forward rate can be described by the equation:

$$-(da/dt) = k_+a^2$$

and after integration

$$1/a - 1/a_0 = k_+t$$

where a_0 is the initial molar concentration of either reactant and k_+ is the forward rate constant. From the plot shown in Figure 3, $k_+ = 26\ M^{-1}\ s^{-1}$. This value is consistent with the value obtained from calculation of the initial reaction rate ($k_+ = 25\ M^{-1}\ s^{-1}$) for the first 90 min of the 1:1.3 molar ratio data at 0.21 mg/mL shown in Figure 2C using the two-component, second-order rate equation:

$$\frac{1}{a-b}\ln\frac{b(a-x)}{a(b-x)} = k_+t$$

where a and b are the reactant molar concentrations and x is the amount of complex formed. At subsequent time points, the reverse reaction becomes significant, and deviation from a second-order reaction is observed. A reasonable estimate of the forward reaction can be made at 23 °C as shown in Figure 4. The forward rate constant can be determined from the initial slope of this plot, yielding an average value of $k_+ = 144 \pm 9\ M^{-1}\ s^{-1}$.

Direct measurement of the reverse rate constants was not feasible due to experimental limitations since it was not possible to "freeze" either the forward or the reverse reaction. Attempts to isolate complexes by HPLC followed by monitoring the dissociation step were unsuccessful since invariably substantial dissociation occurred during collection and pooling and the opposing reaction quickly contributed to the reaction rate.

Evaluation of Thermodynamic Parameters for Single-Site Head-to-Head α–β Association. Association constants for αI–β complexes were determined from replicate samples at equilibrium using the equation:

$$K_a = [\alpha\mathrm{I}\beta]/[\alpha\mathrm{I}][\beta]$$

Samples were incubated over a range of times to ensure that equilibrium was reached at each temperature. Immediately after incubation, samples were separated and quantified using the HPLC gel filtration assay. The average K_a's at multiple temperatures between 0 and 37 °C are listed in Table II. A plot of ln K_a versus $1/T$ shows a linear relationship over the entire temperature range (Figure 5). The slope and y intercept lead to an enthalpy value of –65 kJ mol^{-1} and an entropy value of –0.11 kJ mol^{-1} K^{-1} (Table II). This slope is quite different from that determined for dimer–tetramer association by Ungewickell and Gratzer (1978) which is shown by the dashed

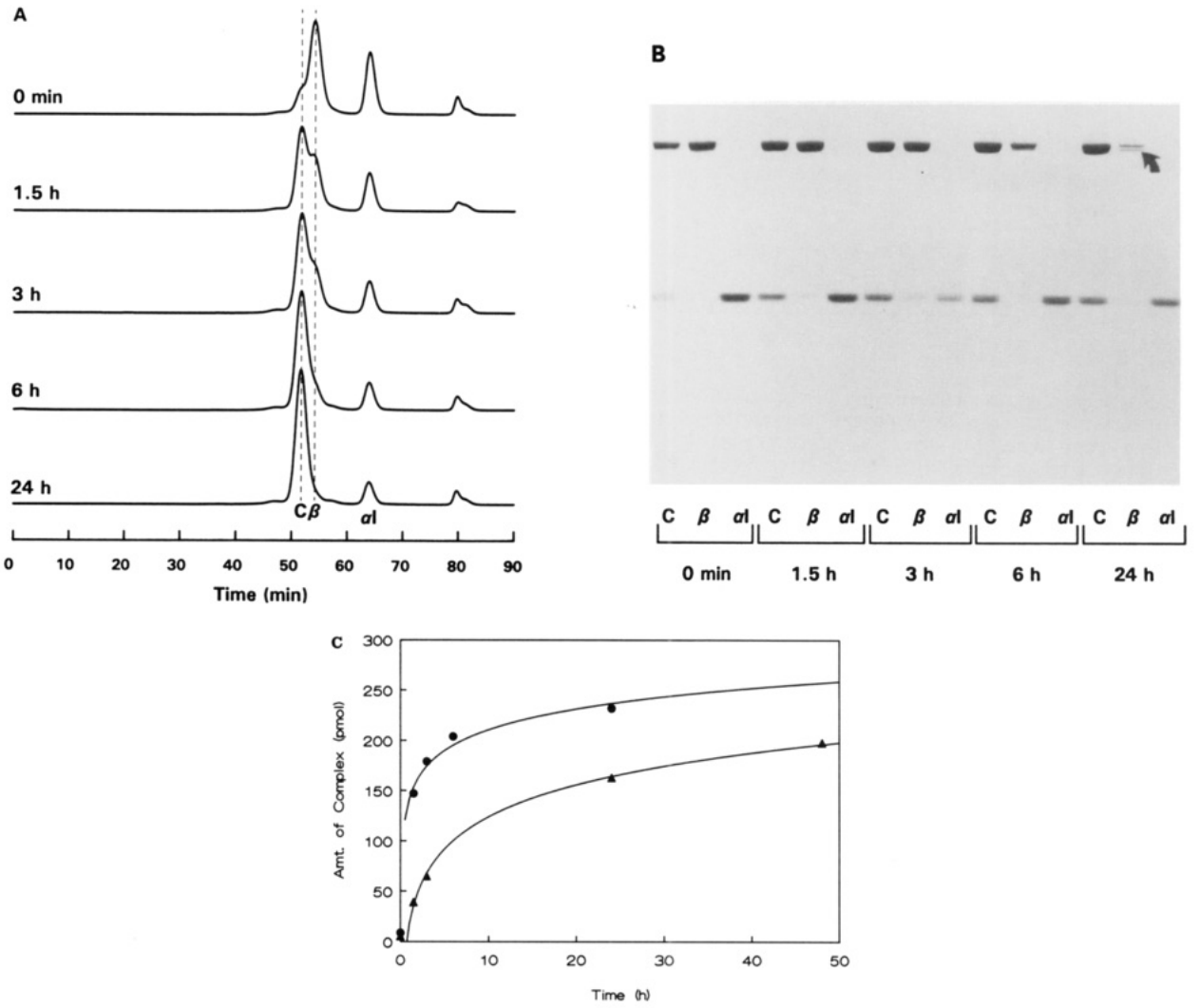

FIGURE 2: Head-to-head association of the αI domain and β subunits at 0 °C. β monomers and the αI domain were combined in a 1:1.3 molar ratio, respectively, on ice. (A) At the indicated times, 90-μg aliquots at a final concentration of 1.15 mg/mL were separated by HPLC gel filtration. The positions of β monomers and β–αI complexes (vertical lines) and the αI domain are indicated. The "0"-min time actually represents about 45 s between mixing and injection onto the HPLC. (B) Coomassie blue stained 7% SDS gel of peak fractions from the separations shown in (A): C, complex; β, β monomer; αI, αI domain peptide. The arrow indicates a minor proteolytic product of the β subunit that cannot associate with the αI domain. (C) Effect of concentration on the rate of head-to-head complex formation. The amount of complex formed versus time of incubation at 0 °C was determined by integration of the complex peaks from the experiment shown in (A) [(●) 1.15 mg/mL] and a similar time course at a lower concentration [(▲) 0.21 mg/mL]. Data points represent single measurements.

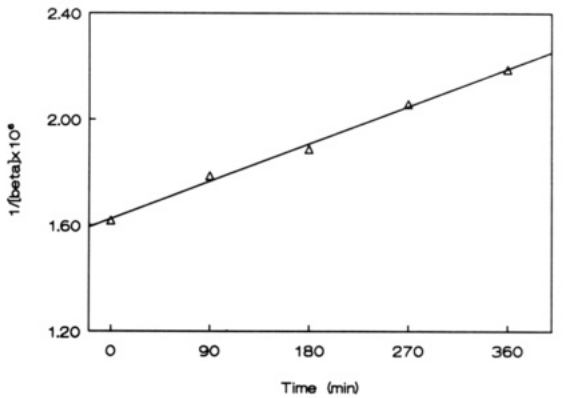

FIGURE 3: Kinetics of αI domain and β monomer association to form a single-site head-to-head complex at 0 °C. Equimolar amounts of αI and β monomers were mixed at a final concentration of 0.20 mg/mL. Complex formation was measured at the indicated times using the HPLC assay. The data illustrated for free β monomer follow a second-order rate plot over the illustrated time course. Data points represent single determinations.

line in Figure 5. As noted above, our measurements of K_a's for dimer–tetramer equilibria using the HPLC assay are consistent with Ungewickell and Gratzer's results using velocity sedimentation which indicates the two methods are in close agreement.

Energy Scheme Relating αI–β Association with Dimer–Tetramer Interconversion. Activation energies for the forward and reverse reactions of the single-site–single-site interaction can be estimated using the Arrhenius equation:

$$\Delta E_a = \frac{RT_1T_2}{T_1 - T_2} \ln \frac{k_1}{k_2}$$

where k_1 and k_2 are either the forward (k_+) or the reverse (k_-) rate constants at the respective temperatures, $T_1 = 273$ K and $T_2 = 296$ K, and R is the gas constant. Reverse reaction rate constants were calculated from the relationship:

$$K_a = k_+/k_-$$

yielding $k_- = 5.8 \times 10^{-6}$ s^{-1} at 0 °C and $k_- = 3.4 \times 10^{-4}$ s^{-1} at 23 °C. The resulting activation energies (Figure 6A) lead to an enthalpy of association of –69 kJ mol^{-1} which is in close agreement with the calculated enthalpy from the van't Hoff plot of –65 kJ mol^{-1}.

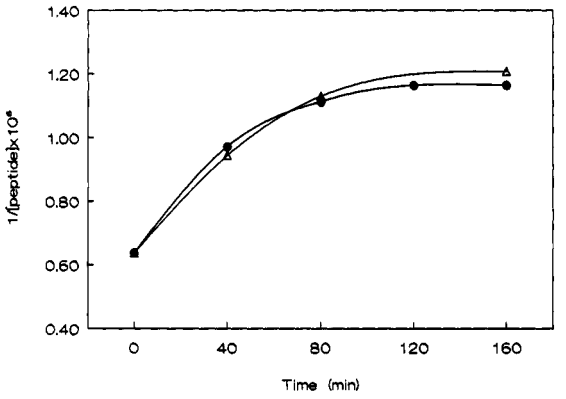

FIGURE 4: Kinetics of αI domain and β monomer association at 23 °C. Equimolar amounts of αI and β monomers were mixed at a final concentration of 0.50 mg/mL. The HPLC columns were at 23 °C, and the flow rate was increased to 0.80 mL/min for this experiment. At the indicated times, aliquots were injected from a single reaction mixture to eliminate pipeting variations. Complex formation was measured at the indicated times using the HPLC assay: (△) β monomers; (●) αI domain. The data illustrated show deviation from a second-order rate plot as the reverse reaction becomes significant. The forward rate constant was estimated from the initial slope. Data points represent single determinations.

Table II: Thermodynamic Parameters for the αI Domain and β Monomer Equilibrium

temp (°C)	K_a (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (kJ mol^{-1} K^{-1})
0	4.5×10^6	−34.7		
10	1.3×10^6	−33.6		
23	4.2×10^5	−32.1	−65	−0.11
30	2.6×10^5	−31.4		
37	1.4×10^5	−30.6		

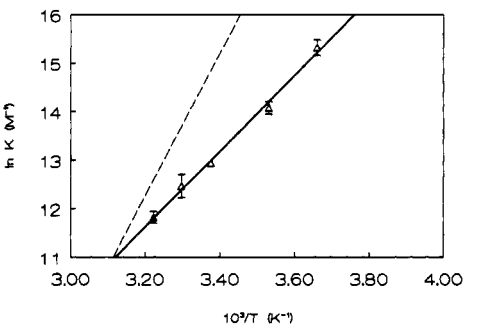

FIGURE 5: Temperature dependence of spectrin head-to-head equilibrium (van't Hoff plot). (△) Temperature dependence of association constants for αI domain–β monomer equilibrium at pH 7.4 and physiological ionic strength. (- - -) van't Hoff plot for dimer–tetramer equilibrium as previously determined (Ungewickell & Gratzer, 1978).

Assuming that the two univalent components, αI and β monomers, are approximately equivalent to two open dimers, the single-site activation energies can be incorporated into an overall energy scheme for dimer–tetramer interconversion as shown in Figure 6B. To develop this model, dimer–tetramer enthalpy values and overall activation energies for dimer–tetramer forward and reverse reactions were taken from Ungewickell and Gratzer (1978). The scheme in Figure 6B indicates that opening the closed dimer is the major contributor to the high activation energy of the forward reaction and opening the first site of the tetramer is the major contributor to the activation barrier for the reverse reaction. This general scheme is consistent with the observed facile interconversion of αI domain and β monomers with the αI–β complex which readily occurs at 0 °C.

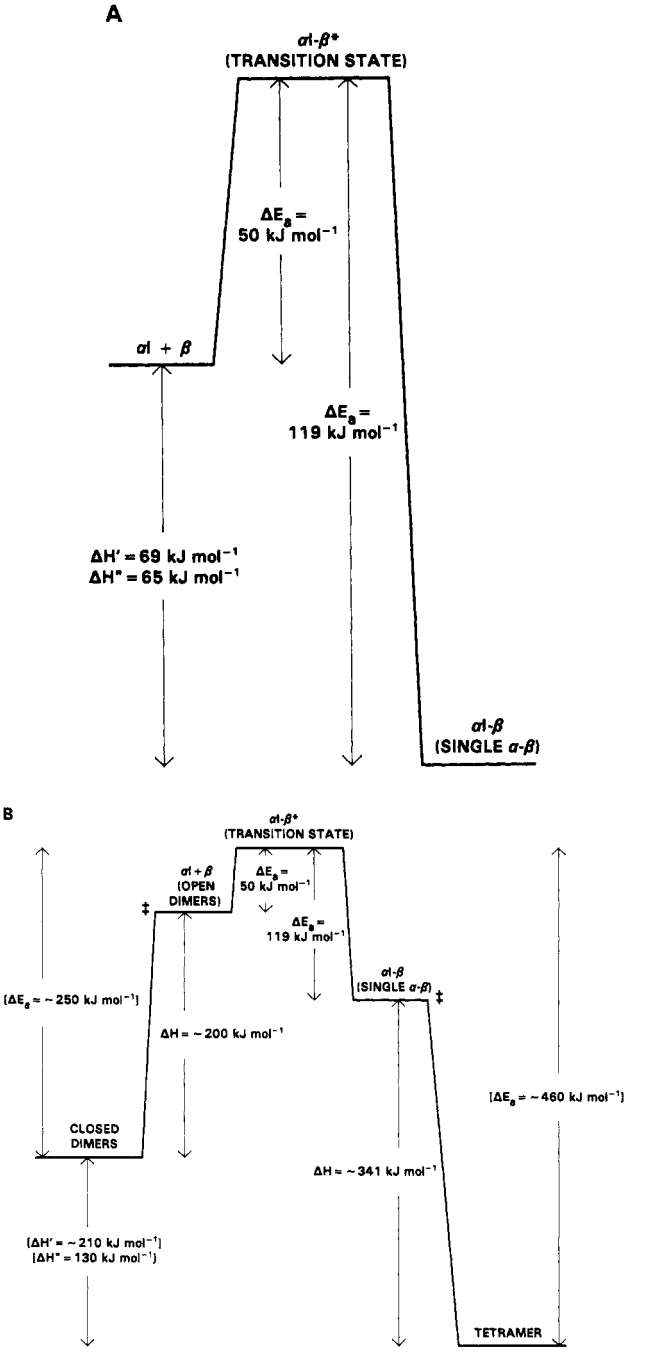

FIGURE 6: Relationship between single-site–single-site association and dimer–tetramer interconversion. (A) Activation energy diagram for single-site–single-site (αI–β) association. $\Delta H'$ is calculated from the difference between forward and reverse activation energies. $\Delta H''$ is from the van't Hoff plot. (B) Diagram illustrating estimated energy states for dimer–tetramer interconversion incorporating the contribution of single-site–single-site interactions. Values in brackets are from Ungewickell and Gratzer (1978) and describe dimer–tetramer equilibrium. $\Delta H'$ and $\Delta H''$ as defined in (A). (‡) Probable transition states between closed and open dimers as well as between single α–β complexes and tetramers cannot be estimated and are not illustrated.

DISCUSSION

As noted above, a closed-loop form of dimers is suggested by electron microscopic images which frequently show looped structures at the physical ends of the rods (Shotton et al., 1979; Morrow & Marchesi, 1981). A reversible opening of the dimer (Morris & Ralston, 1989) or a similar conformational rearrangement (Ungewickell & Gratzer, 1978) has been proposed on the basis of thermodynamic evaluations of tetramer formation. This report provides direct evidence for

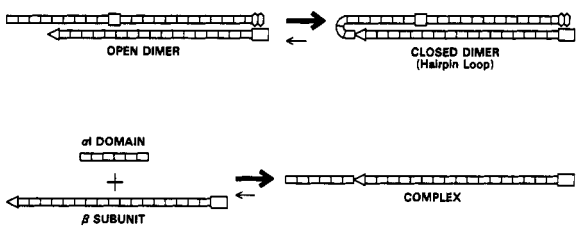

FIGURE 7: Comparison of single-site–single-site head-to-head association with open and closed dimers. The structural motifs of the antiparallel α and β subunits are schematically represented: rectangles, repetitive segments (repeats with an eight-residue insert have an added triangle on their sides); squares, src SH-3-type segment; diamonds, EF hands; large rectangles, actin binding domain; triangles, nonhomologous phosphorylated region. At lower temperatures, both illustrated equilibria are shifted to the right.

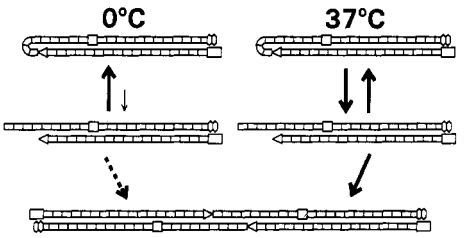

FIGURE 8: Model of dimer–tetramer interconversion at low and high temperatures. The role of the open-loop dimer as a high-energy intermediate between closed-loop dimers and tetramers is illustrated. At lower temperatures, the much higher binding affinity of the α–β site dramatically decreases the concentration of this high-energy intermediate.

opening of a closed dimer as the temperature-dependent conformational change required for tetramer formation. Removal of the side-to-side associated partner of a heterodimer eliminates the thermal barrier for interconversion and extends evaluation of α–β head-to-head binding to lower temperatures. The association of the αI domain and β monomers studied here should be essentially equivalent to formation of the first head-to-head association in the dimer since either one of these two single-site peptides interacts with normal dimers with a similar affinity to dimer–dimer association.

Our observations using two univalent peptides are consistent with a previous report of low-temperature association of univalent mutant dimers (Morris et al., 1989). However, in this previous study, association constants and kinetic parameters were not determined due to the slow analysis time of agarose gels and uncertainty of the mutant spectrin concentrations. In contrast, the purified peptides used in our study could be precisely quantitated, and the HPLC gel filtration assay allowed more rapid analysis times which should minimize errors in reaction rate measurements. Although the elution time of the αI-β complex was about 51 min using a flow rate of 0.40 mL/min at 0 °C, further association of the reactants during the HPLC separation would be minimal since the more slowly migrating αI domain peptide is completely separated from the rapidly migrating β monomer and αI-β complex in about 5 min. Similarly, dissociation of the complex during the separation is apparently minimal since observed molar decreases of the well-resolved αI domain peptide were in good agreement with molar decreases of the β monomer peak and corresponding molar increases in the complex peak. The more rapid reaction rate at 23 °C required a shorter analysis time to make a reasonable estimate of the initial reaction rate (Figure 4). By increasing this flow rate to 0.80 mL/min, the reactants were effectively separated from each other several minutes after injection. Also, as shown in Figure 4, decreases in the αI domain and the β monomer were essentially identical, indicating that little dissociation of complex (a net shift into the β peak) occurred on the time scale of this separation.

Since the two spectrin subunits have a dissimilar number of repetitive motifs, the N-terminal of the longer α subunit probably folds back upon itself to form a hairpin loop involving most of the αI domain (first six repetitive motifs). It then binds head-to-head with the C-terminal region of the β subunit as shown in Figure 7. It is likely that the structure, but not the thermodynamic properties, of this internal head-to-head α–β association closely resembles the head-to-head αI–β association. The closed $\rightleftarrows$ open dimer equilibrium probably roughly approximates αI–β association–dissociation with two obvious differences. First, steric strain contributed by the hairpin loop of the closed dimer does not occur in the αI–β equilibrium. The possible existence of a hairpin in the αI–β complex can be eliminated since the hydrodynamic properties of the head-to-head αI–β complex are consistent with a fully extended structure as illustrated in the lower panel of Figure 7. This steric factor in the closed dimer, relative to αI–β association, is opposed by an entropy factor since the two subunits are sequestered next to each other in the dimer. The relative contributions of these two opposing factors on the closed $\rightleftarrows$ open dimer equilibrium remain unknown, and methods for direct detection of the open and closed dimer species must be developed. However, it should be noted that the cratic entropy component for this reaction can be estimated from $R \ln (1/[H_2O])$ and is quite small (–0.03 kJ mol^{-1} K^{-1}), which indicates that this entropy component plays a minor role in the closed $\rightleftarrows$ open dimer equilibrium. By using the association behavior of the αI and β monomers as a guide, it should now be possible to predict conditions where dimers are predominantly open or closed to facilitate development of appropriate assays for the different dimer forms.

Extrapolation of the strong αI–β association at low temperatures to closed $\rightleftarrows$ open dimer equilibria (with the limitations noted above) predicts that dimers should exist primarily in the closed form at 0 °C. If closed dimers predominate at low temperatures together with a high energy of activation for opening the hairpin loop, little association to tetramers would occur on a realistic time scale (Figure 8). In contrast, when αI and β are mixed, there is no preexisting closed form, and therefore there is no temperature constraint for association. Similarly, at higher temperatures (30–37 °C), dimers must be partially, but not completely, in the open conformation since the assembly kinetics are slower than for the association of two univalent peptides where no closed loop exists.

As noted earlier, the association constants involving dimer associations with either univalent peptides or other dimers, measured by different laboratories using different methods, agree remarkably well (see Table I). However, discrepancies have been reported for thermodynamic parameters of the dimer–tetramer association, the contributions of conformational strain, and the binding affinities of higher order structures beyond tetramers. Ungewickell and Gratzer (1978) originally determined an enthalpy of –130 kJ mol^{-1} and an entropy of –0.32 kJ mol^{-1} K^{-1} for the dimer–tetramer equilibrium. This same laboratory more recently determined that association constants beyond tetramers were about 10–20-fold lower than for dimer–tetramer equilibria (Shahbakhti & Gratzer, 1986). In contrast, Morris and Ralston (1989) evaluated spectrin self-association using a cooperative isodesmic model and determined that association constants for higher order oligomers beyond tetramers are only about a factor of 2-fold lower than for dimer–dimer association. Using this isodesmic model, an enthalpy of –65 kJ mol^{-1} and an entropy

of –0.097 kJ mol^{-1} K^{-1} were obtained for the dimer–tetramer equilibrium (Ralston 1991). As noted above, our measurements of dimer–tetramer association using the HPLC assay are consistent with the thermodynamic parameters determined by Ungewickell and Gratzer (1978) and not with the values obtained from the isodesmic model. It should also be noted that the enthalpy (–65 kJ mol^{-1}) and entropy (–0.11 kJ mol^{-1} K^{-1}) for the univalent–univalent association determined here are essentially identical to the values determined by Ralston (1991) for the spectrin dimer–tetramer equilibrium which would not be expected.

The current energy scheme for the overall dimer–tetramer equilibrium (Figure 6B) has several quantitative limitations. First, the accuracy of the activation energies estimated for the dimer–tetramer equilibrium by Ungewickell and Gratzer (1978) was limited by the narrow temperature range available for determination of rate constants, as noted by those authors. Also, dimers probably exist at all available temperatures as mixtures of open and closed dimers in unknown proportions. The starting point for dimer association to form tetramers is therefore probably not entirely the closed dimer state depicted in Figure 6B. It is also possible that open dimers are not equivalent to the two single-site peptides (αI and β monomers), although this correlation seems reasonable since these peptides, as well as dimers with truncated β subunits, only show about a 2-fold decrease in binding affinity (see Table I). It seems likely that dimers with truncated β subunits cannot form the closed hairpin loop but would have any normal side-to-side interactions that might indirectly affect binding at the tetramer site. Despite these quantitative limitations, the current model provides a useful framework for further analysis of the tetramerization site. More precise analysis of the closed $\rightleftarrows$ open dimer equilibrium would be facilitated by development of methods to directly detect and measure the open and closed forms.

This study directly determines the contribution of the first α–β head-to-head association and dissociation to dimer–tetramer interconversion. Opening the closed-loop dimer or the first site on a tetramer is the major thermodynamic barrier for spectrin dimer–tetramer interconversion. Removing this constraint frees the reaction from the normally observed temperature barrier and substantially increases the rate of association. Further studies are in progress to locate the actual sites on the α and β subunits involved in head-to-head interactions and to evaluate the rate of dimer hairpin loop closure.

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