

Influence of Lateral Association on Forced Unfolding of Antiparallel Spectrin Heterodimers*

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Protein extensibility appears to be based broadly on conformational changes that can in principle be modulated by protein-protein interactions. Spectrin family proteins, with their extensible three-helix folds, enable evaluation of dimerization effects at the single molecule level by atomic force microscopy. Although some spectrin family members function physiologically only as homodimers (e.g. α -actinin) or are strictly monomers (e.g. dystrophin), α - and β -spectrins are stable as monomeric forms but occur physiologically as α,β -heterodimers bound laterally lengthwise. For short constructs of α - and β -spectrin, either as monomers or as α,β -dimers, sawtooth patterns in atomic force microscopy-forced extension show that unfolding stochastically extends repeats ~ 4 – 5 -fold greater in length than native conformations. For both dimers and monomers, distributions of unfolding lengths appear bimodal; major unfolding peaks reflect single repeats, and minor unfolding peaks at twice the length reflect tandem repeats. Cooperative unfolding thus propagates through helical linkers between serial repeats (1, 2). With lateral heterodimers, however, the force distribution is broad and shifted to higher forces. The associated chains in a dimer can stay together and unfold simultaneously in addition to unfolding independently. Weak lateral interactions do not inhibit unfolding, but strong lateral interactions facilitate simultaneous unfolding analogous to serial repeat coupling within spectrin family proteins.

As cytoskeletal proteins, spectrin superfamily proteins play important roles in cell organization and membrane mechanics (3, 4). As flexible linkers that bind to actin filaments, these proteins function in both monomeric and associated forms. Erythroid α I- and β I-spectrin were the first identified spectrins among a still growing superfamily that includes α -actinin, dystrophin, utrophin, and many additional proteins that share homologous triple-helical repeat motifs (5). In the red cell, α -chains of 20 repeats associate antiparallel along their lengths with β -chains of 17 repeats. The association is nucleated near the N terminus of β -spectrin (6) that ends with an actin binding domain, which mediates formation of a cross-linked network (7). The ability of spectrin monomers to first dimerize laterally

and then associate head-to-head as tetramers that cross-link actin is especially crucial to the resilience of the red cell membrane in circulation. Defects in association, for example, lead to membrane instabilities typical in hereditary pyropoikilocytosis (8). Like red cell spectrin, α -actinin, with its four homologous repeats, also associates laterally and cross-links actin, imparting stability to structures that range from focal adhesions to Z-lines of myotubes (5). Dystrophin and utrophin (5), in contrast, are hypothesized to impart cortical stability to diverse membranes as monomeric actin-binding proteins.

For all of the spectrin family proteins, length and extensibility are deemed central to function. Surprisingly, perhaps, recent single-molecule studies (1, 9, 10) show that individual spectrin repeats will unfold under modest tensile forces. This is consistent with such motifs contributing directly to cytoskeletal flexibility and strain softening (11). When compared with a growing list of forcibly unfolded protein domains, primarily stressed by atomic force microscopy (AFM)¹ methods (12), the triple-helical fold of spectrin already appears to be among the most facile to unfold. Mechanical unfolding of spectrin raises higher order questions, however, such as the effect of association between chains.

To specifically address the effect of the lateral association state of spectrin on unfolding, we have applied the AFM method of single molecule mechanics to two- and four-repeat constructs of α - and β -spectrin with comparison to the laterally associated α,β -heterodimer (see Fig. 1). The α -actinin homodimer and an eight-repeat rod segment of dystrophin were also studied. A variety of solution studies on spectrin dimerization have been conducted in the past (7, 13). The dissociation constant (K_d) for the β_{1-4} and α_{18-21} dimer complex was measured to be ~ 10 nM compared with the ~ 10 pM reported for α -actinin dimers (14, 15). Furthermore, the affinity of β_{1-2} for α_{21-20} was found to be much stronger than that of β_{3-4} for α_{19-18} (13) thus dividing the lateral dimer complex into a strong end and a weak end (Fig. 1C). Although other AFM studies have elaborated RNA hairpin interactions (16) and insulin monomer-monomer association/dissociation interactions (17), none to date have explicitly examined the inhibiting effects, if any, of protein dimerization on domain unfolding. Spectrin dimerization is predicated on the typical range of protein interactions: hydrophobic sequestration, hydrogen bonding, and most importantly electrostatic interactions between paired triple-helical bundles (13). Here we probe these dimeric interactions through their effects on flexibility and unfolding. Evidence of positive cooperativity in forced unfolding of serial repeat interactions emerged in our recent work on spectrin that has been extended to temperature effects (1, 2).

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¹ The abbreviations used are: AFM, atomic force microscopy; pN, piconewton.

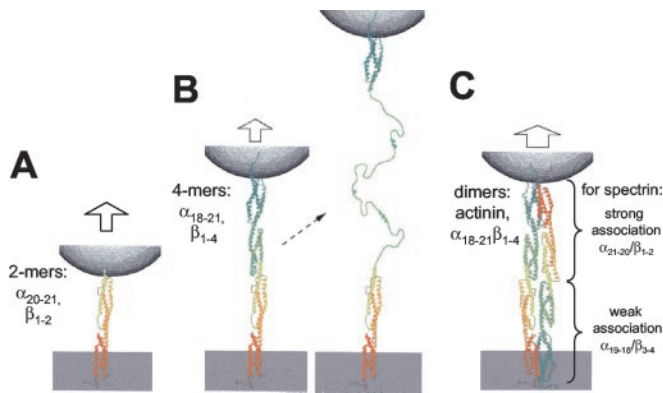


FIG. 1. Forced extension schematic for various spectrin and actinin constructs: two-repeat constructs α_{20-21} and β_{1-2} (A), four-repeat constructs α_{18-21} and β_{1-4} (B), and eight-repeat $\alpha_{18-21}\beta_{1-4}$ -spectrin heterodimer and actinin homodimer (C). Illustrated is the structural layout for the spectrin heterodimer. The $\alpha_{20-21}/\beta_{1-2}$ lateral interaction was found to be much stronger than that of $\alpha_{18-21}/\beta_{3-4}$, thus forming a strong and weak affinity component within the dimer complex. The actinin homodimer has a homogeneous affinity throughout its laterally associated chain, and its association constant is much greater compared with the spectrin heterodimer. The imposed rate of extension for all of the experiments was 1 nm/ms.

We examine here whether lateral interactions facilitate or oppose unfolding. By a thorough statistical analysis, we conclude that strong lateral interactions lead to coupled unfolding of laterally adjacent repeats, whereas weak lateral interactions are surprisingly neutral in their effects on the force to unfold a repeat.

MATERIALS AND METHODS

Protein Preparation—The four N-terminal repeats of β I-spectrin (β_{1-4}), the last four repeats of α I-spectrin (α_{18-21}) (see Table I), and the four-repeat actinin central domains were expressed recombinantly and prepared as described (6). Truncated α - or β -spectrin constructs exist only as monomers in solution (*i.e.* no homodimers), whereas actinin exists only as a tight homodimer. Stable $\alpha\beta$ -heterodimer was purified from mixed monomers by gel permeation chromatography in phosphate-buffered saline as described elsewhere (18). Protein was kept on ice for AFM studies and typically was used within hours. Immediately before use, any protein aggregates were removed by centrifugation at $166,000 \times g$ at 2°C for 60 min, and dynamic light scattering was used to verify monodispersity prior to experiments.

An AFM experiment was begun by adsorbing 100 μl of 0.03–0.1 mg/ml protein for 15 min at room temperature onto either freshly cleaved mica or amino-silanized glass coverslips. The surface was then rinsed lightly with phosphate-buffered saline and placed without drying under the head of the AFM; all measurements were carried out in phosphate-buffered saline. Lower protein concentrations generated minimal AFM results; higher protein concentrations showed higher unfolding forces, proving consistent with the results below indicating that domains in multiple, parallel chains are forced to unfold all at once. Fluorescence imaging of labeled protein demonstrated homogeneous adsorption, and AFM imaging after scratching the surface showed that no more than a monolayer of molecules covered the substrate (as illustrated in Ref. 19).

Dynamic Force Spectroscopy—Two AFMs were used with similar results: (i) a Nanoscope IIIa Multimode AFM (Digital Instruments, Santa Barbara, CA) equipped with a liquid cell and (ii) an Epi-Force Probe from Asylum Research. Sharpened silicon nitride (Si_3N_4) cantilevers (Park Scientific, Sunnyvale, CA) of nominal spring constant $k_C = 10$ piconewtons (pN)/nm were commonly used, with equivalent results obtained using 30 pN/nm cantilevers. k_C was measured for each cantilever using the manufacturer's directions at each temperature, and additional calibrations were performed as described previously (19). For any one temperature, thousands of surface-to-tip contacts (~ 4000) were collected and later analyzed with the aid of a semi-automated, visual analysis program custom written in C++. Because protein-unfolding events are stochastic, and the experiment is intrinsically random in many ways, collecting and analyzing thousands of peaks is necessary to provide an accurate statistical survey of the unfolding possibilities.

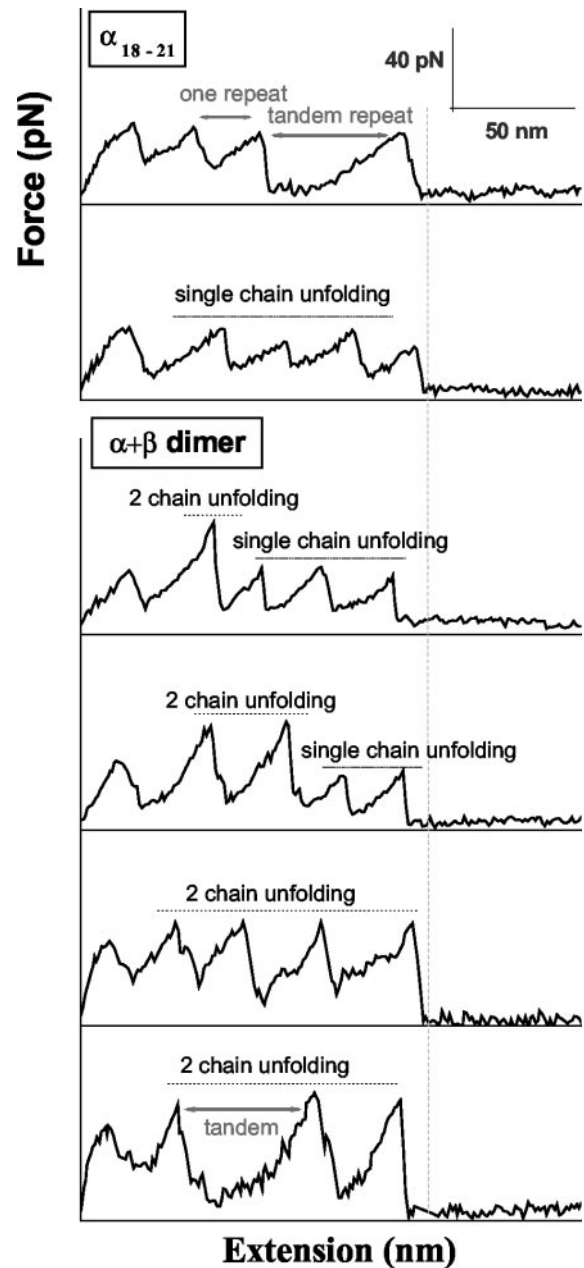


FIG. 2. Force-extension spectrograms for α_{18-21} -spectrin and heterodimers of $\alpha_{18-21},\beta_{1-4}$ -spectrin. Both one- and tandem-repeat unfolding processes occur with the latter characterized by at least one peak-to-peak length that is approximately twice that of single repeat unfolding events. Peaks in the sawtooth patterns are characteristic of unfolding and show, consistent with random attachment, a varied number of peaks, $N_{\text{pk}} = 4$ in *topmost spectrogram*. All of the spectrograms shown have either 4 peaks with a tandem repeat or 5 peaks with all single repeat events, yielding a similar total unfolding length. The peak average of the dimers is approximately twice as large in force compared with that of the monomers, suggesting two adjacent chains unfolding simultaneously. The *last dimer spectrogram* shows a combination of large peaks (two chains unfolding) and small peaks (single chain unfolding). The larger peaks often occur before the smaller peaks, contradicting the findings of Li *et al.* (20).

Initial results at the beginning of an experiment lasting many hours were similar to those obtained at the end of the experiment. Thus nearly all of the data was analyzed.

RESULTS AND DISCUSSION

Force-Extension Curves Analysis—AFM-imposed extension of α - or β -spectrin chains, either as pure monomers or as premixed $\alpha\beta$ -dimers, generally leads to an asymmetric saw-

tooth pattern of force peaks (Fig. 2). The ramped portions of the force-extension curves beyond the first peak correspond to the extension of an unfolded domain up to the point where another (still folded) domain in the chain unfolds. The number of peaks in an extension curve, N_{pk} , would seem to reflect predominantly which repeat in the chain the randomly applied AFM tip physisorbs to on contact. N_{pk} thus provides a useful measure of how many domains are subject to extension. The sawtooth patterns here with α -spectrin monomers reproduce and extend our recent findings (1) for β -spectrin by frequently showing extra wide intervals between force peaks. These are signatures of tandem repeat unfolding events. The heights of the force peaks with spectrins are invariably found as elsewhere (1, 2, 9, 10) to be less than ~ 50 pN, which is much smaller than the forces of 150–300 pN reported for titin under similar rates of protein extension (0.01–10 nm/ms) (12). Lower forces are likely to be indicative of more facile unfolding of proteins *in vivo* (11).

Heterodimer Force-Extension Curves—Lateral heterodimers of α_{18-21} plus β_{1-4} contain eight spectrin repeats in total (Fig. 1C). Their force-extension spectrograms are remarkably similar to those of the four-repeat α_{18-21} -spectrin (Fig. 2). The 4-peak spectrograms with one extra wide tandem repeat event yield a similar total unfolding length to the various 5-peak spectrograms with all single repeat events. Histograms of length distributions discussed later will quantitate such results more thoroughly. As tentatively suggested in Fig. 2, the peaks of the heterodimer appear on average to be of almost twice the force as the predominant force peaks of the monomer. This indicates that the two adjacent chains of the heterodimers will unfold synchronously. A more thorough statistical analysis discussed below will again substantiate this assertion. The dimer chains could also split apart during unfolding and display a combination sometimes seen of both large high force peaks (two chains unfolding) and small low force peaks (single chain unfolding); the bottommost spectrograms of Fig. 2 illustrate this. In such cases, the larger peaks almost always occur before the smaller peaks. Such results contrast with findings of Li *et al.* (20) for single titin chains of alternating I²⁷ and I²⁸ domains and are discussed in detail later.

Total Unfolding Length and Peak Distribution Analysis—Four thousand tip-to-surface contacts were typically performed on $\alpha + \beta$ -spectrin heterodimers as well as on all of the two- and four-repeat α - and β -spectrin monomer constructs. The total unfolding length and the peak distribution results are summarized in Fig. 3. It is immediately clear that each construct yielded different $\max(N_{pk})$ values. The two- and four-repeat α -monomers, like the two- and four-repeat β -monomers, show a $\max(N_{pk}) = 4$ and 6, respectively (1). Such results indicate that the first and last peaks are desorption processes as widely assumed (1, 2). The results also imply that partial unfolding events are not likely to occur with the spectrin constructs here, which contrasts with the studies of Lenne *et al.* (10) on chicken brain spectrin. The first peak and the force of the last peak were therefore ignored in the analyses and spectrograms with two peaks or less were not cumulated in force (see Fig. 7) and length (see Fig. 5) distributions. Spectrograms of 0–2 peaks accounted for $>80\%$ of the data because of the low concentrations of protein dispersed on the substrates in these experiments. As concluded by others (1, 21), single molecule experiments must be “designed to fail” more often than succeed to ensure the experiment is a single molecule experiment. Only the few $N_{pk} \geq 3$ spectrograms are protein unfolding events, and they ensure that we are operating in the single molecule limit.

For each N_{pk} , Fig. 3B shows the average total unfolding length (\pm S.D.) as calculated beyond the second peak. Heterodimer results for N_{pk} differ remarkably little from those of

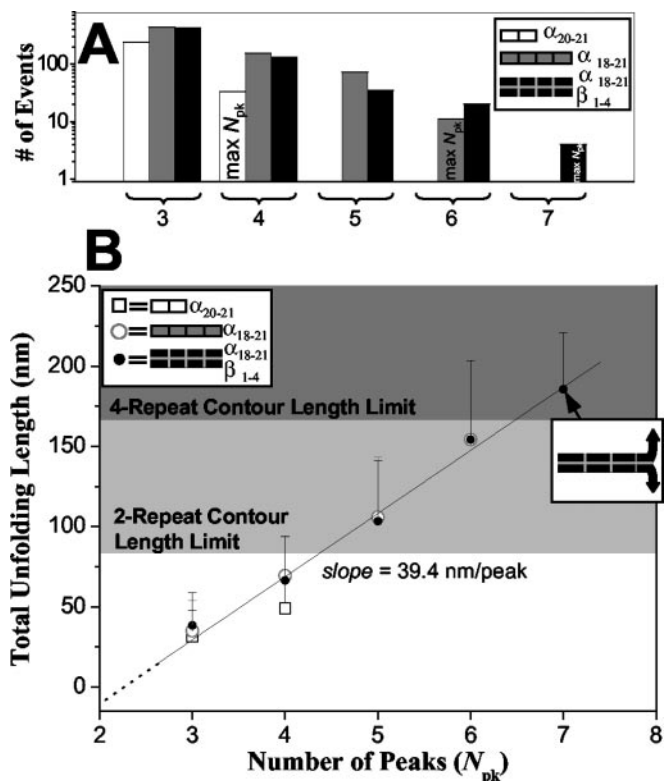


FIG. 3. Force-extension spectrogram statistics for N_{pk} and total unfolded length. A, distributions of N_{pk} for the $\alpha + \beta$ -spectrin dimer as well as the two- and four-repeat α -spectrin constructs after 4000 contacts with surface-adsorbed protein. B, average (\pm S.D.) total unfolding length for each N_{pk} is obtained from the sawtooth extension curves beyond the second peak. The light gray and dark gray regions correspond to generic limits of extension calculated from the number of amino acids (Table I) for the fully stretched contour length, L_c , of either the two- or four-repeat α -spectrin constructs, respectively. The slope of the best-fit line through all of the data is the average distance between peaks accounting for both single- and tandem-repeat unfolding processes.

the monomers. A few 7-peak extension curves were counted in heterodimer experiments (Fig. 3A) and were not present in monomer results. Except for these few 7-peak heterodimer curves (4 total), all other spectrograms fall within the relevant contour length limits for either two repeats (transition to light gray) or four repeats (transition to dark gray). This finding is certainly consistent with the lack of any higher order spectrin oligomers or aggregates in solution (see “Materials and Methods”). Because the $\alpha\beta$ -heterodimer consists of eight repeats, the rare seventh peak might correspond to an infrequent splayed chain as suggested earlier. Splaying is further illustrated in Fig. 3B from the overextension of the $N_{pk} = 7$ spectrograms beyond the four-repeat contour length limit. The best-fit line through all of the data has a slope that averages both single and tandem events. The linearity of the unfolding length versus N_{pk} plot is remarkable given the large extensions probed and the monomer versus dimer systems studied. Knowing that the length of the four-repeat rod domain of α -actinin is about 25 nm, the unfolding length for these homologous constructs is readily calculated to be up to 6–7-fold longer. Domain unfolding thus visibly modulates extensibility.

Spectrin Heterodimer Versus an Eight Repeat in Series Dystrophin Construct—Another spectrin family protein (dystrophin) expressed in separate studies as a construct with eight repeats in series (22, 23) offers an important contrast to the $\alpha\beta$ -heterodimer with its eight repeats distributed as 4 in-series between two laterally associated chains. The serial versus lateral difference is most clearly illustrated in a plot of $\max(N_{pk})$

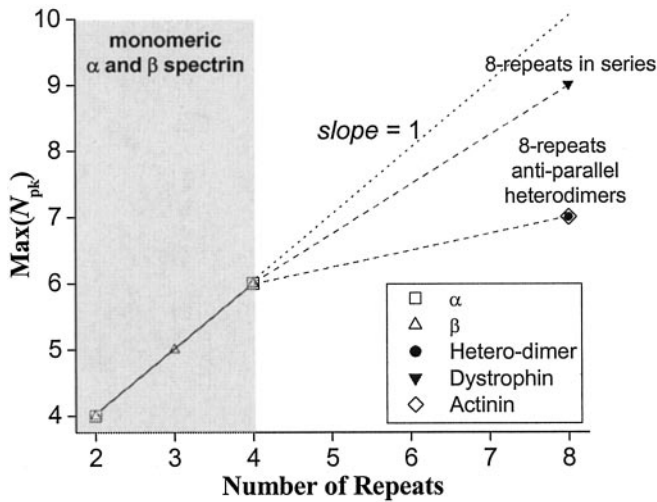


FIG. 4. Dependence of N_{pk} histogram characteristics on the number of repeating domains. The plot of $\max(N_{pk})$ versus the number of spectrin repeats is shown. For small α and β constructs, a linear relationship of unit slope is found. For the eight repeats in series (dystrophin), a $\max(N_{pk})$ of 9 is found,² whereas a maximum of only 7 peaks is observed for both the eight-repeat spectrin heterodimers and actinin homodimer, which are both antiparallel complexes.

versus the number of repeats (Fig. 4). The $\max(N_{pk}) = 7$ for the spectrin heterodimer clearly departs from the linear trend of slope = 1 for the various monomers of spectrin α and β . The heterodimer result also contrasts with the eight-repeat dystrophin construct in showing a $\max(N_{pk}) = 9$ that is close to the expected $\max(N_{pk}) = 10$.² Because the N_{pk} distribution is known to decay exponentially (1, 19) the likelihood of unfolding all eight serial spectrin repeats in dystrophin (to give 10 peaks) may be statistically too rare to encounter even with >3000 contacts, especially given the increased occurrence of tandem unfolding events (1, 2). Nonetheless, consistent with the spectrin heterodimer here, an eight-repeat antiparallel actinin homodimer also showed $\max(N_{pk}) = 7$. The difference in $\max(N_{pk})$ between the construct with eight repeats in series and the two complexes with eight repeats laterally associated clearly indicates a mechanism wherein repeats in both chains of a given dimer will often unfold simultaneously rather than splay apart to a wishbone conformation and then unfold as eight repeats in series.

Peak-to-Peak Unfolding Length Distribution Analysis—Histograms of the unfolding lengths between peaks (l_{pk-pk}) for the four-repeat α -spectrin monomer and the spectrin heterodimer are shown in Fig. 5. The distributions do not appear significantly dependent on the number of peaks in an extension curve, which seems consistent with a random desorption process. Both the monomer and heterodimer histograms appear bimodal with principal means differing by a factor of 2 (1, 2). For α -spectrin, each peak is composed of four gaussian curves whose means reflect known proportions for domain contour lengths of the constructs (see Table I). The spectrin heterodimer was fitted with just two gaussian curves of the same width (w), which proved to be similar to those of the monomer.

The averages of the major peaks range from 22 to 27 nm, including results for β_{1-4} -spectrin (1, 2). Lenne *et al.* (10) found a similar length for a 4-mer of a single spectrin repeat flexibly linked in series. Data for actinin homodimer appear similar to that for the spectrin heterodimer, showing a bimodal distribution with a factor of ~ 2.0 between the mean for the major and

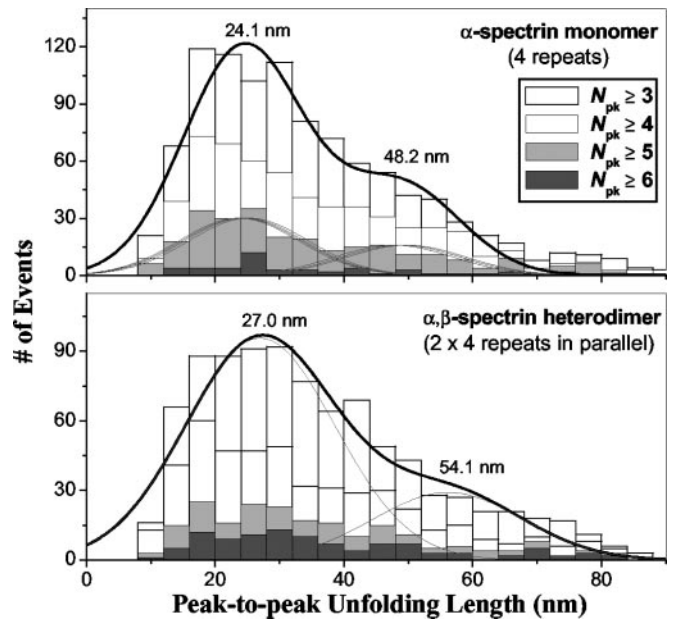


FIG. 5. Histograms of peak-to-peak unfolding lengths for saw-tooth extensions of monomeric α_{18-21} -spectrin and α,β -spectrin heterodimer. For the α -spectrin monomer, the major peaks were fitted with sums of gaussian peaks that reflect proportional contour lengths for single repeats (see Table I). The minor peaks were likewise fitted but with contour lengths of tandem repeats. The spectrin dimer was fitted with two gaussian peaks of the same width ($w = 16$ nm), which prove similar to those of the monomer ($w = 13$ nm). The overall sum of all of the gaussian peaks is indicated by the heavy black line; a factor of about two (1.9–2.1) between the major and minor peaks is apparent for all.

TABLE I
Primary structure properties of spectrin constructs
Domain and total contour lengths, l_c and L_c , respectively, have been calculated with a peptide length of 0.37 nm/aa.

Domain	No. of aa ^a	Contour, l_c
		nm
α_{21}	111	41.0
α_{20}	114	42.1
α_{19}	107	39.5
α_{18}	109	40.2
Extra	9	3.3
Total, L_c		166.1
Average		41.5
β_1	120	44.3
β_2	114	42.1
β_3	109	40.2
β_4	106	39.1
Extra	2	0.7
Total, L_c		166.4
Average		41.6

^a aa, amino acid.

minor peaks (data not shown). Because the major peaks in the unfolding distributions correspond to unfolding a single repeat, the freely fitted factor of 2.0 in mean length between the first and second peaks provides a strong indication of the unfolding of a tandem pair of in-series repeats as proposed in Fig. 1. Consistent with this interpretation, the $N_{pk} = 6$ subhistogram for the α -monomer shows that the vast majority (90%) of the l_{pk-pk} fall within the major envelope of single repeat unfolding events; $N_{pk} = 6$ thus corresponds to four single repeat events plus initial and final desorption events. The few events outside of the single repeat envelope evidently reflect overextension of the cumulated slack that develops toward the end of a long extension before final desorption terminates the spectrogram (1). The same observation also applies to the $\alpha\beta$ -heterodimer.

Because the lengths of the three helices composing each

² R. Law, G. Liao, H. Zhao, L. Sweeney, and D. Discher, manuscript in preparation.

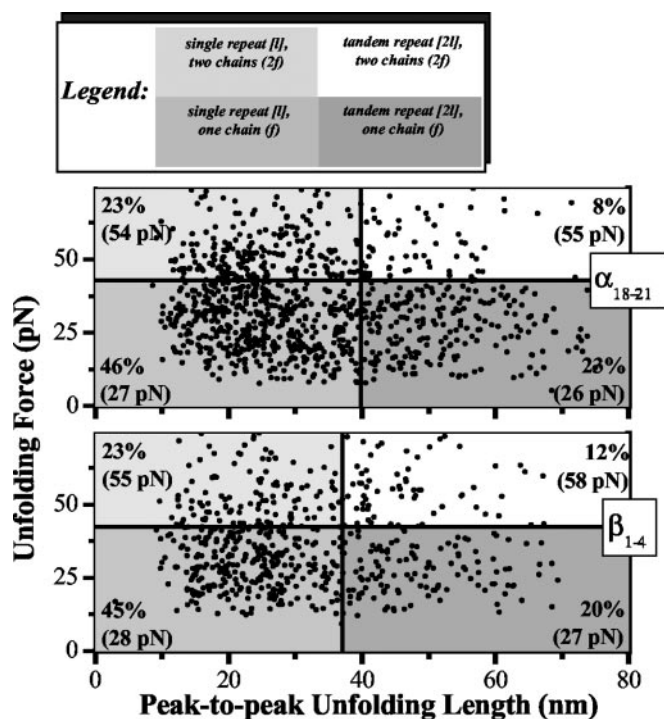


FIG. 6. Scatterplots of unfolding force versus unfolding length. Dividing lines are obtained from the intersection between the major and minor gaussian peaks of Figs. 5 and 7 and thus represent a stringent, if simplistic, separation of unfolding processes or states. The legend at the top represents the simplest interpretation of these states in terms of unit unfolding length, l , and unit unfolding force, f , of a single repeat. The lower two quadrants correspond to single-chain unfolding, both single- and tandem-repeat unfolding, as indicated. The fraction of data points within each of the four quadrants is indicated as a percentage of total data points together with the quadrant-average force in parentheses.

repeat are known from crystal structures to be 4–5 nm long or 12–15 nm/repeat, the single repeat unfolding lengths measured as 21–27 nm must involve some helix unwinding but are still much less than the ~ 40 nm contour lengths of each repeat. This indicates that an unfolded repeat is not completely stretched before the unfolding of the next domain occurs. The implicit solvent molecular dynamics of Paci and Karplus (25) indeed suggest that helices can persist in unfolding as sketched in Fig. 1. Such a result is consistent with both broader distributions and lower forces for unfolding spectrin in comparison, for example, to titin whose unfolding lengths approach domain contour lengths at 5–10-fold higher forces. In this context, the fact that the $\alpha\beta$ -heterodimer here gives longer peak-to-peak lengths (27 and 54 nm) than the constituent monomers suggests higher forces are likely involved in unfolding with dimer, as shown below.

Tandem Repeat Unfolding Events—The frequency of tandem repeat unfolding events in the $\alpha\beta$ -heterodimer is visibly lower relative to single repeat events. The spectrin heterodimer shows the fewest tandem events as a percentage of total events, just 20% for heterodimer (area under 54.1 nm peak) compared with 40% (48.2 nm peak) for the α -monomer. This suggests that tandem unfolding does not propagate as well in two laterally associated heterodimer chains compared with a single chain.

Unfolding Force Distributions Reveal Spectrin Heterodimers—Force distributions for unfolding monomers also appear bimodal with gaussian means differing again by a factor of ~ 2 , but scatterplots are critical to first clarifying the mechanisms of unfolding. By pairing a given unfolding length with the appropriate unfolding force, a scatterplot (Fig. 6) demon-

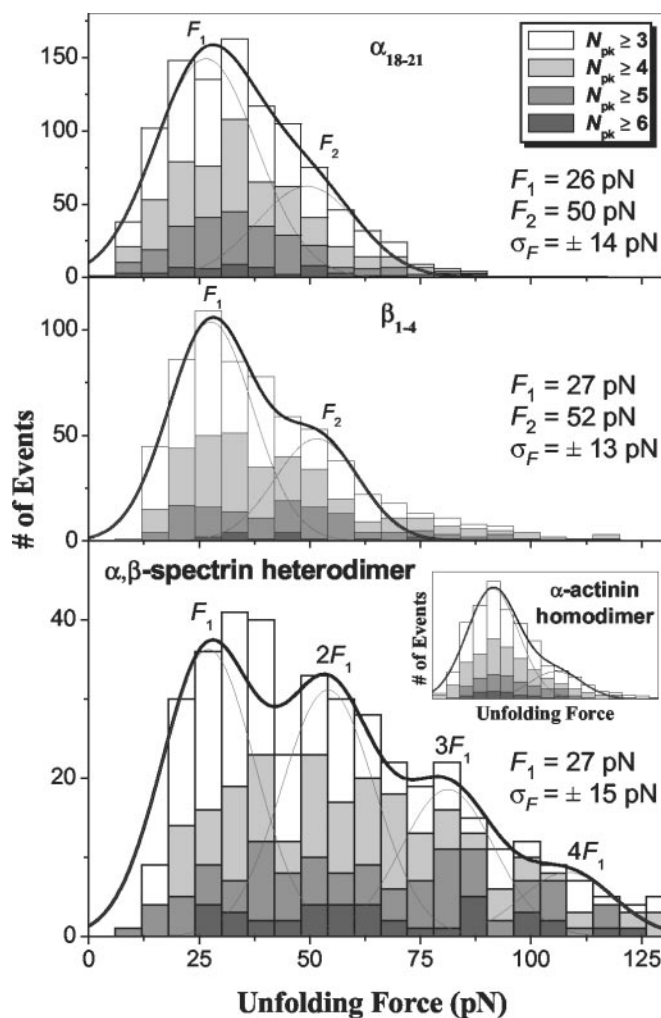


FIG. 7. Histograms of unfolding forces for sawtooth extensions of monomeric α_{18-21} -spectrin, β_{1-4} -spectrin, and $\alpha\beta$ -spectrin heterodimer. The bimodal histograms of the monomers were fitted with two gaussian peaks of the same width, and the overall sums of the gaussian peaks are indicated by heavy black lines. Spectrin heterodimer results were fitted with four gaussian peaks. For the first gaussian peak (see Table II) the averages of the major gaussian peaks from the two constituent monomers were used (denoted as F_1) along with their averaged width (w) as restricted parameters. For the second gaussian peak, twice the major peak value ($2F_1$) was used. For the third gaussian peak, three times the major peak value ($3F_1$) was used, and the fourth gaussian peak was four times the major peak value ($4F_1$). In contrast to the spectrin heterodimer, the unfolding force distribution for the 2×4 repeat α -actinin homodimer can be best fitted with a bimodal gaussian distribution (inset).

strates that factors of 2 in force for α - and β -monomers (Fig. 7, F_1 and F_2) reflect pulling on one chain versus two (or perhaps a loop in one chain).

For the heterodimer, the force distribution (Fig. 7) is considerably broader, more multi-peaked, and shifted to relatively higher forces than the force distributions of the monomers. Four distinct peaks are suggested for the heterodimer, indicative of four different unfolding processes. The monomer results with $F_1 \approx 27$ pN were used in fitting with four gaussian curves centered at iF_1 ($i \in \text{integer}$). For the second gaussian, two times the major peak value ($2F_1$) was used and so on. The average width (w) was also parameter-restricted to be close to the average widths of the monomers. Only the relative amplitude of each peak was varied in the fitting of duplicate experiments (Table II). Such a fitting seems to capture the four distinct peaks extremely well compared with freely fitting the data with four gaussian curves. In contrast to the spectrin

heterodimer, the unfolding force distribution for the α -actinin homodimer fits best with a bimodal gaussian distribution. Unfortunately, the α -actinin monomer quickly associates into a homodimer, making unfolding studies on the monomer impossible. No comparison of forces for the actinin monomer to forces for the actinin dimer can therefore be made. Perhaps future mutants of a non-dimerizing but folded α -actinin will make such studies possible.

Heterodimer Unfolding Pathways—First, just as unfolding

TABLE II

Force histogram gaussian parameters for spectrin heterodimers

The force and amplitude from the four gaussian curves represent the unfolding properties for single, two, three, and four monomer chains.

Peak	Force	Amplitude					
	<i>pN</i>						
F_1 : single chain	27	36.2 ± 1.8					
$2F_1$: two chains	54	$3F_1$: three chains	81	18.6 ± 1.7	$4F_1$: four chains	108	8.1 ± 1.7
$3F_1$: three chains	81	18.6 ± 1.7					
$4F_1$: four chains	108	8.1 ± 1.7					

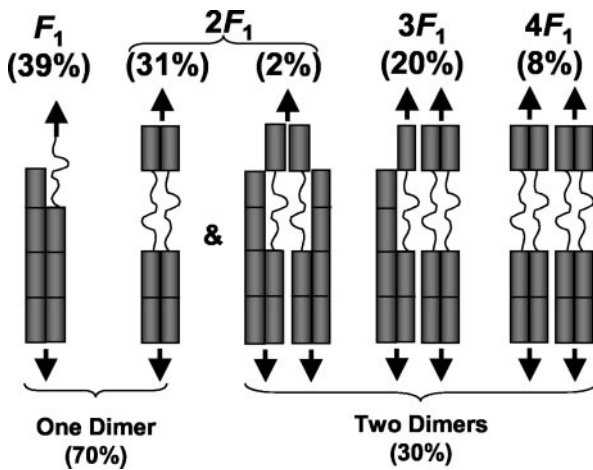


FIG. 8. Possible dimer unfolding scenarios and frequency of events. The dimer unfolding scenarios represented in the four gaussian distributions (Fig. 7) along with their frequencies are illustrated. The percentages were calculated using Table II and by assuming that the dimers follow the same unfolding patterns of monomers (70% single-chain and 30% double-chain events) from Fig. 6.

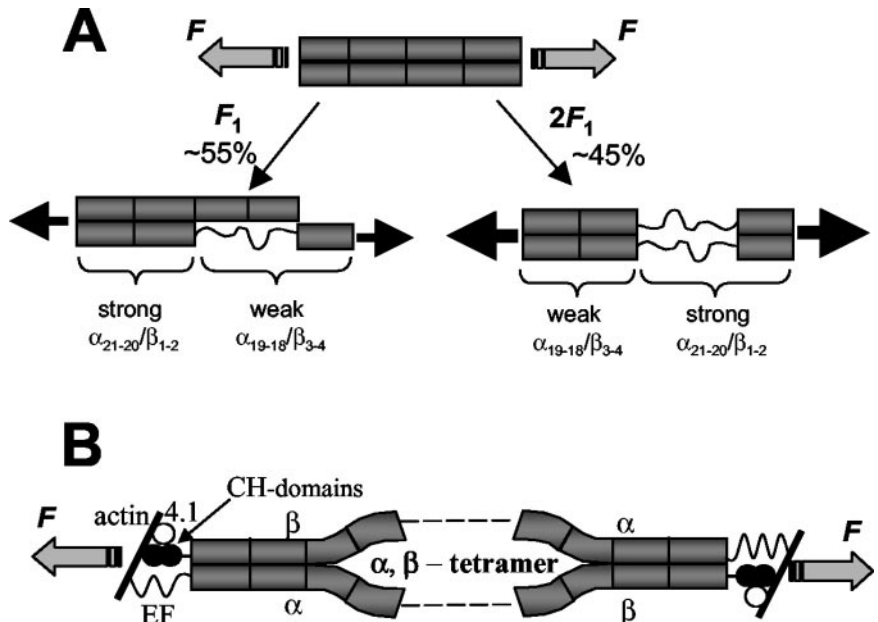
two independent chains is inevitable in monomeric spectrin studies (1), pulling on two heterodimers is also to be expected and seems apparent from the third and fourth gaussian peaks. The multimodal fit indicates multiple pathways of unfolding. The 27-pN peak reflects the unfolding of a single chain in the dimer as it dissociates from its partner. The force needed to unfold such repeats in a single chain is remarkably unaffected by the adjacent chain. Such scenarios are possible but certainly not at the exclusion of also frequently unfolding both chains of the heterodimer to give the second gaussian peak. The minimal influence of the two chains on unfolding in each may again seem surprising but is consistent with the first peak. This may be overly simplified, because the force needed to split apart the heterodimer interaction may be incorporated in the first peak that is skewed beyond the gaussian fit of 27 pN. Freely fitting the force histogram of the heterodimer yields a first gaussian peak at 31 pN (not shown). The difference of 4 pN from the free fit and the forced fit (from monomer components) could be the *small* shearing force needed to split the dimer apart during unfolding. Accounting for both processes plus their two-chain counterparts would certainly tend to broaden the cumulated force distributions as found.

All of the further heterodimer unfolding scenarios are presented in Fig. 8. The frequency of each scenario can be estimated using results from the α - and β -monomers. The force distributions for both α - and β -monomers displayed about $\sim 70\%$ single-chain and $\sim 30\%$ double-chain unfolding events (Fig. 6, lower versus upper quadrants). Assuming that experiments on the heterodimer has the same one- versus two-chain frequency and using the respective gaussian heights in Table II, one can readily calculate the frequencies for all of the unfolding scenarios sketched. Note that in the second dimer gaussian peak of Fig. 7, $2F_1$ is composed of unfolding (i) two chains within the same dimer and also (ii) two single chains from two distinct heterodimers. From the calculations summarized in Fig. 8, the first scenario (i) appears to be much more frequent (31%) compare with the latter (ii) scenario (2%). Similarly, unfolding possibilities from three or more unassociated dimers are probably too insignificant to consider relative to the high frequency of the unfolding of one or two dimers.

Based on the overall statistics of Fig. 8 for pulling on one dimer, we conclude that there is an almost equal probability of the AFM tip unfolding a single chain (55%) within a dimer as

FIG. 9. Unfolding pathways *in vitro* and forces on the native dimer chains.

A, single-chain versus two-chain unfolding within a dimer *in vitro*. Calculations based on Fig. 8 show that 55% of the time, only one chain within the dimer unfolds at a force F_1 . The other 45% of the time, both chains within the dimer unfold at a force $2F_1$. The attachment of the AFM tip to either the weak or strong end of the dimer complex determines one- or two-chain unfolding, respectively. **B**, schematic of the force, F , on a tetrameric spectrin cross-link between F-actin junctional complexes. The force is taken up by both chains of the heterotetramer. Thermal fluctuations at the molecular scale will tend to make the division unequal, biasing unfolding processes toward unfolding in one chain rather than two.



there is of unfolding both chains (45%) within a dimer (Fig. 9). Recalling that the $\alpha_{20-21}/\beta_{1-2}$ lateral interaction is much stronger than that of $\alpha_{18-19}/\beta_{3-4}$, this strong *versus* weak association within the dimer complex explains why a single chain unfolds just as frequently as both chains when the AFM tip has an equal possibility of attaching to either end of the heterodimer. Attaching to the weaker end will result in the unfolding of single chains, whereas attaching to the stronger end will result in the simultaneous unfolding of both chains within the dimer. This is not the case for the α -actinin homodimer, which has the same affinity throughout its laterally associated chain. In addition to its more homogenous inter-chain affinity, the association constant of α -actinin is much greater compared with the spectrin heterodimer, consistent with the bimodal distribution for unfolding force in Fig. 7.

The two chains of the heterodimer may come apart during unfolding, because dimeric interactions rely heavily on complementary electrostatic interactions within the triple-helical dimer interface (13). These might be easily severed with modest shearing force well below that experienced at the molecular level by the red cell in the circulation (26). As shown in Fig. 2, the splaying of dimers can also occur partway through the unfolding process such that both chains may initially unfold together synchronously, then one chain may split off and continue to unfold, whereas the other partially folded chain remains folded. This raises the question as to how spectrin or related proteins are stressed *in situ* (Fig. 9B). In both erythroid and non-erythroid systems, the N-terminal CH-domains of β -spectrin bind actin (27), and β -spectrin can also bind protein 4.1 into a highly stable ternary complex with F-actin (24). The EF-hands of α -spectrin might also enter into this complex (27) and effectively supplement the stress-sharing because of the strong lateral interaction between α_{21} and β_1 repeats. When F-actin junctional complexes are separated, the $\alpha\beta$ -tetramer that interconnects two adjacent complexes will be stressed by a force, F , that will generally be divided by the α - and β -chains. As the force increases in time to $F \sim F_1$, fluctuations may make the division of force so unequal that one repeat in one of the chains will unfold (i). The slack in that chain will almost immediately lead to a load transfer to the antiparallel chain and cause unfolding in it (1). If F is divided more equally between the two chains of a spectrin tetramer, then the force could increase to $F = 2F_1$ and unfold laterally adjacent repeats that might or might not be in registry (ii). Of course, only the actin-binding ends of the spectrin heterodimer associate laterally with any high affinity. Even so, the expectation is that scenario (i) is more frequent than (ii) in cells, and a hint of that emerges in the *in vitro* results (Fig. 9A).

Conclusion—The extensible unfolding of monomeric *versus* heterodimeric spectrin is compared here by AFM. The results demonstrate that the two chains in the heterodimer could

either stay intact and unfold simultaneously or splay apart and unfold as a single chain, because little force is needed to sever at least some heterodimeric interactions given the strong *versus* weak ends of the heterodimer complex. The basic method used here was to fit force distributions for heterodimers based on the separate results for the component monomers. Comparisons reveal various dimer unfolding scenarios as well as frequencies of occurrence. From these statistics, both chains within the dimer unfold just as frequently as a single chain within the dimer unfolds at half the force as the two chains. The results thus suggest that, in contrast to cooperativity in tandem repeat unfolding, lateral interactions do not strongly facilitate or oppose single-repeat unfolding processes in spectrin heterodimer extension.

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