Stability of the Dystrophin Rod Domain Fold: Evidence for Nested Repeating Units

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ABSTRACT An examination of fragments of the human dystrophin rod domain, corresponding to a single structural repeating unit, showed that a critical chain length, defined with a precision of one residue at the C-terminal end, is required for formation of the native tertiary fold. We report here that extending the chain by six residues beyond this minimum results in a large increase in conformational stability. This is not related to a change in association state of the polypeptide. The results support the conjecture that successive repeating units in the rod domain of the spectrinlike proteins form a nested structure, in which the *N*-terminal part of the three-helix bundle of one repeat packs into the overlapping structure of the preceding repeat. This would be expected to affect functional characteristics related to flexibility of the dystrophin rod domain.

INTRODUCTION

Dystrophin is a highly elongated protein, with a contour length of ~175 nm (Pons et al., 1990). Most of this length is contributed by the central rod domain, which comprises 25 degenerate repeating units with sequences homologous to those of the 106-residue repeats of erythroid spectrin (Koenig et al., 1988; Davison and Critchley, 1988). The precise function of dystrophin is a matter of debate, but in its absence the capacity of the membrane to withstand mechanical stresses is greatly impaired (Petrof et al., 1993), its osmotic fragility is increased (Hutter et al., 1991), and myotubes exhibit a large diminution in stiffness (Pasternak et al., 1995). It therefore seems likely that dystrophin exerts a regulatory effect on the mechanical properties of the sarcolemma, qualitatively similar to that of spectrin in the red-blood-cell membrane.

If the spectrinlike proteins are elastomers (Elgsaeter et al., 1986; Markin and Kozlov, 1988) with end-to-end separations constrained by the spacing of the protein network junctions, then the elasticity will be governed by the flexibility of the polypeptide chain and its end-to-end distance in the lattice. The hydrodynamic properties of spectrin are those of a wormlike coil, resembling, for instance, DNA (Reich et al., 1982; Stokke et al., 1986), rather than of a broken rod. This would imply that the flexibility is an intrinsic feature of a relatively uniform structure and does not arise from the presence of flexible linkers between rigid structural elements. The structural repeats are composed of three-stranded α-helical-coiled coils ~5 nm long (Yan et al., 1993), and it has been conjectured that successive repeats make up a continuous, nested structure (Cross et al., 1990; Yan et al., 1993). We show here that extension of a single repeat, corresponding to the minimum folding unit (Kahana and Gratzer, 1995), by a few residues into the sequence of the adjoining repeat leads to a large increase in negative free energy of folding in a manner consistent with expectation for a nested structural model.

Received for publication 27 February 1996 and in final form 3 June 1996.

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0006-3495/96/09/1605/06 $2.00

MATERIALS AND METHODS

The dystrophin repeat selected for study was the second in the rod domain. This is one of the closest in sequence similarity to the erythroid spectrin repeats (Cross et al., 1990). Fragments with a common *N* terminus (residue 439 in the dystrophin sequence) and 119 and 123 residues long, respectively, were obtained by expression in *E. coli*. The preparation of the first of these fragments (F119) has been described (Kahana et al., 1994; Kahana and Gratzer, 1995). The second (F123) was expressed similarly, by use of DNA obtained by PCR from dystrophin cDNA, kindly provided by Professor K. E. Davies. The forward primer corresponded to nt 1523–1546, and the reverse primer to nt 1870–1891. The identity of the product was checked by *N*-terminal sequencing. As before, the protein was recovered as inclusion bodies from the *E. coli* expression strain. These were dispersed in 6 M guanidinium chloride and 5 mM dithiothreitol and purified by gel filtration in the same solvent, in which the preparations were also stored at −20°C. The protein was then further purified by ion-exchange chromatography on MonoQ in a Pharmacia FPLC apparatus. Samples were applied in 20 mM BisTris, pH 6.1, and eluted with a sodium chloride gradient. The bulk of the protein emerged in a single sharp peak, desorbed at ~0.15 M in the case of F119 and at 0.23 M for F123. The purified material was dialyzed into 0.1 M sodium chloride and 20 mM sodium phosphate, pH 7.6, and used in this buffer for most physical studies.

Circular dichroism was measured in a Jobin-Yvon CD6 instrument in path lengths of 0.2–2 mm. Protein concentrations were determined spectrophotometrically by use of calculated molar absorptivities (Perkins, 1986). For thermal denaturation profiles the temperature in the cell housing was varied in intervals of 5°C between 5° and 75°C. Urea denaturation profiles at 20°C were determined by addition of solid urea to fixed volumes of protein. Urea concentrations and dilution factors were calculated from standard data (Kawahara and Tanford, 1966). In calculating the equilibrium constant for unfolding in the region of the (presumably two-state) transition, we applied a linear correction for the small slope of the profile above and below the transition (Pace and Laurents, 1989). If $[\theta]$ is the molar residue ellipticity thus corrected at a given temperature or urea concentration and those for the folded and unfolded states (above and

below the transition region) are $[\theta]_f$ and $[\theta]_u$, then the equilibrium constant is given by $K = ([\theta]_f - [\theta])/([\theta]_f - [\theta]_u)$.

Molecular weights were determined by sedimentation equilibrium in a Beckman Optima XL-A analytical ultracentrifuge, equipped with absorption optics. Rotor speeds of 24,000 and 28,000 rpm were used at a temperature of 5° or 10°C. The proteins were examined at several concentrations. The liquid column of ~2.5 mm was scanned at 280, or at 230 nm when the protein concentration was sufficiently low. After equilibrium had been reached we increased the rotor speed to 42,000 rpm to achieve meniscus depletion and thus check for the presence of ultraviolet-absorbing material of low molecular weight. For determination of molecular weights a calculated value (Perkins, 1986) of the partial specific volume of 0.728 ml g^{-1} was assumed for both fragments. Sedimentation velocities were measured at 60,000 rpm or in a synthetic boundary cell at 42,000 rpm.

To test for a slow self-association equilibrium, protein samples at a concentration of ~0.15 mg ml^{-1} were incubated for as long as 48 h at 20° and 30°C or for 6 h at 37°C. They were then examined in the native state by gel electrophoresis in 10% polyacrylamide gels in a Tris-Bicine buffer system at pH 8.3, run in the cold, and in some cases by sedimentation equilibrium.

RESULTS

The circular dichroism spectra, measured at 20°C, of the purified dystrophin fragments of 119 and 123 residues (designated F119 and F123) were within error the same, with molar residue ellipticities at the extremum at 222 nm of −25,900 and −26,200 deg cm^2 $dmol^{-1}$, respectively. This corresponds, in the absence of any other contributions than those of the α-helical and unordered states, to a helicity of ~72%, assuming a value for the α-helix of −36,000 (Greenfield and Fasman, 1967). At 5°C the apparent helicity increases to 79%. A multicomponent fit of the spectrum with a basis set of standard proteins (CONTIN computer program, following Provencher and Glöckner (1981)) gives the higher proportion of 86% α-helix, with no β-structure; this is close to the expected value, based on the structure of a single *Drosophila* spectrin repeat (Yan et al., 1993).

Thermal unfolding profiles of F119 and F123 are shown in Fig. 1. Both profiles display a well-defined sigmoid transition, compatible with a two-state process, as observed before for F119 and for the smaller folded fragment, F117 (Kahana and Gratzer, 1995). The striking feature of the profiles is the large disparity in conformational stability between the two polypeptides, reflected by a difference of 17°C in the temperature at the midpoint of the transition, T_m. The gradual diminution in ellipticity with increasing temperature below the transition may indicate the fraying of the helix bundle ends (Holtzer and Holtzer, 1992). Fig. 1 (inset) shows the van't Hoff plots derived from the transitions: these indicate (Table 1) that the additional four residues in F123 cause an increase in the negative enthalpy of folding of some 10 kcal mol^{-1}.

Unfolding profiles generated by addition of urea to aqueous solutions of F119 and F123 are again widely separated (Fig. 2). For quantitative evaluation it is in general justified to assume a linear relation between free energy of folding and urea concentration (Dill, 1985; Santoro and Bolen, 1992), so that $\Delta G°(u) = \Delta G°(w) - m[\text{urea}]$, where the free energies refer to solutions with (u) and without (w) urea and

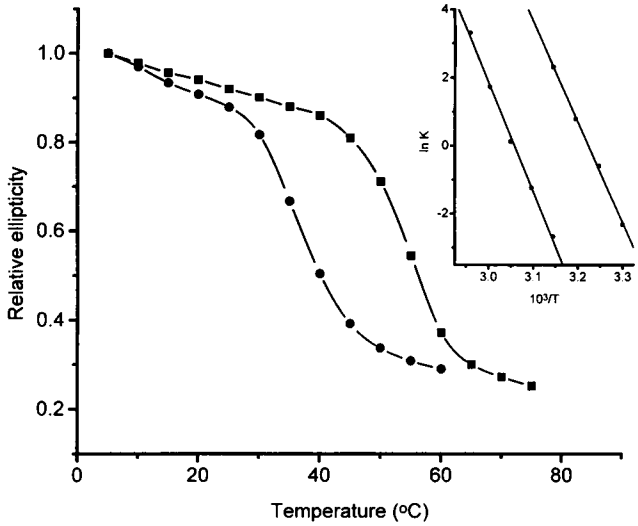

FIGURE 1 Thermal melting profiles, measured by circular dichroism, of dystrophin rod fragments of 119 (●) and 123 (■) residues. Inset: van't Hoff plot derived from melting profiles.

m is an empirical constant. Fig. 2 (*inset*) shows the linear plots, leading to extrapolated free energies of folding in the absence of urea. As before, there is a large difference in conformational stability between F119 and F123, with apparent free energies of folding in aqueous solution of −2.5 and −6.4 kcal mol^{-1}, respectively, at 20°C. These values also agree reasonably well with those derived from the van't Hoff plot (Table 1). As found before (Kahana and Gratzer, 1995), the change in heat capacity on denaturation, $\Delta C_p = (\partial \Delta H/T)_p$, is smaller than can be detected by measurements of the kind made here.

The single repeat of *Drosophila* spectrin, studied by Yan et al. (1993), crystallized as a dimer. This was formed by straightening of the hairpin bend between helices B and C of the three-helix bundle. The bundles in the dimer are thus made up of helices A and B from one subunit and helix C of the other. It must be assumed that this arrangement is favored because the residues normally in the loop between the B and C helices have a preference, or at all events a tolerance, for the α-helical state, so that elimination of the break between two helices outweighs unfavorable factors, such as the entropy change associated with dimerization. To

TABLE 1 Thermodynamic stability of the native conformation of dystrophin rod fragments

Measured thermodynamic property	Fragments	
	F119	F123
Thermal denaturation midpoint, T_m (°C)	37	54
Gibbs free energy of folding at 20° (kcal mol^{-1})*	−3.3	−7.5
Enthalpy of folding (kcal mol^{-1})	−56	−65
Urea denaturation midpoint (M)	1.7	4.4
Gibbs free energy of folding (water) (kcal mol^{-1})#	−3.5	−6.4

*From van 't Hoff plot.
#From linear extrapolation to zero urea concentration.

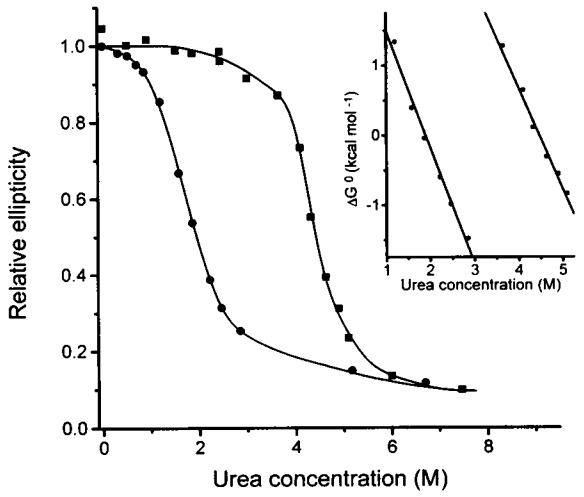

FIGURE 2 Urea denaturation profiles, measured by circular dichroism, of dystrophin rod fragments of 119 (●) and 123 (■) residues. Inset: Extrapolation of folding free energy, derived from denaturation profiles, to zero urea concentration.

determine whether the dystrophin fragments studied here were monomeric or dimeric we examined them by sedimentation equilibrium. An equilibrium distribution for F123 is shown in Fig. 3 *B*. Assuming ideal behavior at this low protein concentration, the sample appears monodisperse with a molecular weight of 15,100 (calculated value for the monomer, 14,900). The freshly prepared fragment F119 also appeared monodisperse or very nearly so, with a molecular weight of 14,400, the calculated value for the monomer.

Storage of samples or incubations for as long as 48 h at 20° and 30°C or 6 h at 37°C led to the appearance of polydispersity in the sedimentation equilibrium of both fragments. An example of this effect is shown in Fig. 3 *A*. For this sample, which had been kept for three weeks on ice, a two-component fit to the distribution was obtained, with a preponderant component (88% of the total) of molecular weight 14,200 and a minor component of 88,000. However, there is no reason to suppose that this is a unique fit, and the presence of a minor proportion of polydisperse aggregates is more likely. Progressive aggregation is in fact to be expected, because we have observed this to occur extensively at high protein concentrations even in the cold (Kahana et al., 1994). To determine whether the fragments are capable of undergoing reversible association to dimer in the manner displayed by the 14th repeat of *Drosophila* α-spectrin (Yan et al., 1993; Ralston et al., 1996), a high-resolution method is to be preferred. If a significant monomer–dimer equilibrium were to prevail in the concentration range of our experiments, interconversion would have to be slow in the cold, inasmuch as sedimentation equilibrium of fresh samples at 5–10°C detects only monomer. This is certainly the case for the *Drososphila* α-spectrin repeat (Ralston et al., 1996). Therefore gel electrophoresis allows resolution of the dimer. Solutions of both fragments at ~0.15 mg ml^{-1}

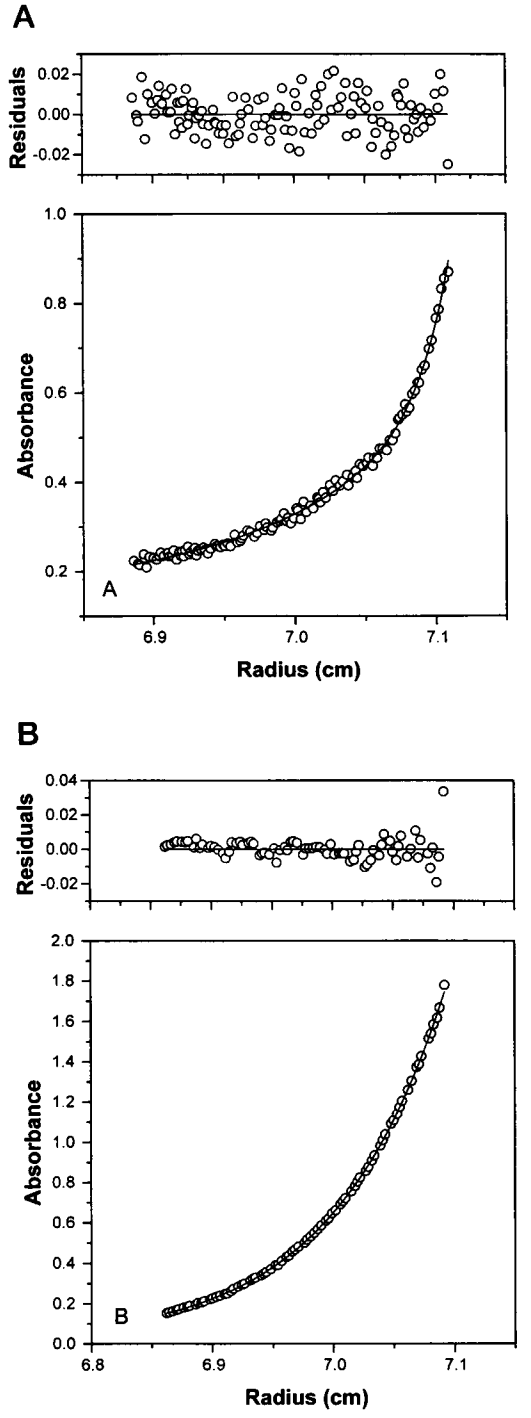

FIGURE 3 Representative sedimentation equilibrium distributions of dystrophin rod fragment preparations: (*A*) Fragment of 119 residues after storage for three weeks on ice. Points are experimental absorbances, and the curve is the calculated best fit for a two-component mixture of monomer, giving 88% of a component of molecular weight 14,200 (calculated value for F119 monomer, 14,400) and 12% of one of 87,600 (assuming a calculated partial specific volume of 0.728 ml g^{-1}). This is unlikely to be a unique fit, and the heavier material is probably a polydisperse aggregate (see text). The heavier component appears to be absent, or nearly so, from fresh preparations. (*B*) Freshly prepared fragment of 123 residues. Points are experimental absorbances, and the line is the calculated best fit for a single ideal solute of molecular weight 15,060 (calculated value for the monomer, 14,900). Residuals are shown in the upper panels.

were incubated at 20 and 30°C for as long as 48 h and at 37°C for 6 h. They were then examined by gel electrophoresis in the cold in the absence of denaturants. None of the samples showed any indication of a dimer zone. Thus significant dimer formation could be excluded, at least under the conditions of the above studies, and any slow monomer–dimer equilibrium, if it exists, would be characterized by an association constant of less than $\sim 10^3$ M^{-1} at any of the incubation temperatures.

The sedimentation coefficient of fresh F123 was also determined and found to be 1.59S, which is again consistent with expectations for a monomeric sample.

DISCUSSION

Our earlier data gave evidence of a close structural (as well as sequence) similarity between the dystrophin rod domain repeat and the spectrin repeats (Kahana et al., 1994). In addition, it proved possible to define to a precision of one residue the minimum extension of the chain in the C-terminal direction required to generate the native tertiary fold (Kahana and Gratzer, 1995). Thus, when the C-terminal residue of the F117 fragment is eliminated, the tertiary structure does not form. A similar all-or-none constraint probably applies also to the *Drosophila* α-spectrin repeat (Winograd et al., 1991). The basis of this phenomenon is not clear, especially because the side chain of the critical C-terminal residue of F117 should be on the outside of the three-helix bundle, remote from other side chains, and can be mutated with no significant change in the stability of the tertiary fold (Kahana and Gratzer, 1995).

We found a small increase in structural stability on extending the chain length of 117 residues by a further 2 residues (Kahana and Gratzer, 1995), but the data shown here reveal a very much larger effect on stability when a further 4 residues are added. Participation of dimer formation in causing this effect can be excluded, and the dimerization of the 14th repeat in the α-chain of *Drosophila* spectrin (Yan et al., 1993; Ralston et al., 1996) may thus be an unusual function of the particular amino acid sequence of the loop between the B and C helices (in the terminology of Yan et al. (1993)). It is less likely to be a general characteristic of isolated repeats, especially because helix-breaking residues, in particular proline and glycine, are abundant in the inferred loop segments throughout the rod domains of these proteins (Winder et al., 1995). In our dystrophin fragments a probable helix-breaking sequence, SSGD, occurs at the beginning of the presumptive loop. Recently Pascual et al. (1996) reported that a chicken brain α-spectrin repeat also generates no dimer, even at very high concentration (1 mM) at 25°C.

The sedimentation coefficient of 1.59S implies a compact structure, falling within the range expected for a native globular protein of the monomer molecular weight (Halsall, 1967). The corresponding anhydrous frictional ratio is 1.26, or 1.13 after correction for hydration, assuming a typical value of 0.3 g g^{-1} bound water. This corresponds in turn to a prolate ellipsoid of revolution with axial ratio 3:1, which is a good approximation of the shape of the *Drosophila* spectrin repeat (Yan et al., 1993). As Ralston et al. (1996) have shown, adequate agreement can also be obtained by a bead model (Garcia de la Torre and Bloomfield, 1977), in which the shape of the particle is approximated by an elongated array of spheres.

A sequence alignment with the *Drosophila* α-spectrin repeat is readily defined, even though the number of identical residues is relatively small (12%, with 42% similarity). It is determined by the multiple alignment for all human erythroid spectrin and dystrophin repeats (Winder et al., 1995), which reveals a number of highly or partly conserved features. With the one three-residue gap, other alignments than that shown in Fig. 4 give marginally higher identity and similarity but fail in the dystrophin repeat to reproduce conserved anionic side chains at three positions in helices A and B and the highly conserved tryptophan in helix A with a flanking hydrophobic residue. The alignment for helix C by contrast is quite unambiguous and shows 25% identity with 58% similarity as well as important conserved features, including an almost invariant tryptophan at C15. The single gap of three residues occurs in a loop, as identified by secondary structure prediction and observed in the *Drosophila* spectrin repeat crystal structure (Yan et al., 1993).

With the alignment shown, the *N* terminus of dystrophin fragments F119 and F123 falls eight residues before that of the *Drosophila* spectrin repeat. This position was chosen for the expressed polypeptides because it corresponded to the *N* terminus of the stable folded fragment, generated by proteolysis of a longer sequence; the chain could thus be assumed to have been cut outside the structured, proteolytically resistant entity (Kahana et al., 1994).

The α-helical segments of the spectrin repeat sequences are characterized by a clear heptad hydrophobic repeat, indicative of the coiled-coil conformation. Even in the much less regular dystrophin repeats, multiple alignment allows the heptad signal to emerge (Cross et al., 1990; Parry and Cohen, 1991; Parry et al., 1992). In F119 the pattern is quite strong, especially in the C helix, with which we are concerned here, and conforms with the alignment. Moreover, it continues through the additional turn of helix in F123, as shown in Fig. 4.

In the structure of the spectrin repeat (Yan et al., 1993) helix B in the helix bundle projects four residues beyond the end of helix C. In modeling the relationship between successive repeats Yan et al. allow the first four residues of helix A (which is continuous with helix C of the preceding repeat) to pack into the bundle with the overlapping residues of helix B. Our observations indicate that the resulting interaction is energetically important.

In a nested structure flexibility must be an intrinsic characteristic of the triple-helical conformation. Loss of a few helical residues from the bundle reduces the stability of this structure, as the above results indicate, thereby (depending

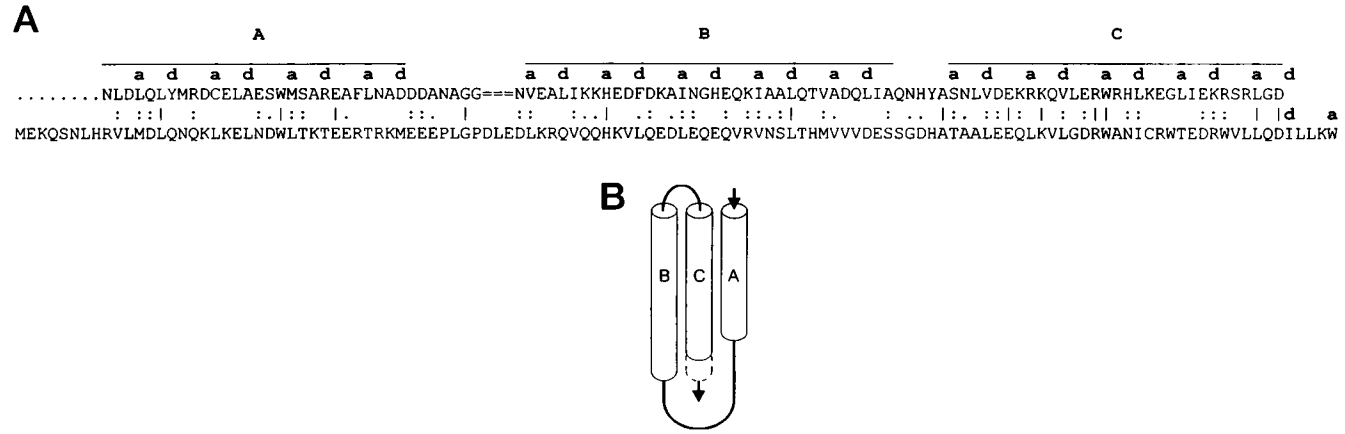

FIGURE 4 Aligned sequences of *Drosophila* α-spectrin repeat 14 (Winograd et al., 1991) (*upper line*) and dystrophin fragment, F123 (*lower line*). The minimal folding unit of the dystrophin repeat terminates at gln-117, one residue before the position corresponding to the C terminus of the crystallized α-spectrin repeat (Yan et al., 1993). The lines denote the α-helices identified in the spectrin fold by Yan et al. and designated A–C. Positions a and d of each heptad repeat, which are preferentially occupied by hydrophobic residues, are indicated. Below is shown in schematic form the helix bundle that makes up the structure of the spectrin repeat (Yan et al., 1993). The dashed segment represents the additional turn of helix in the dystrophin fragment, F123, compared with F119.

on molecular crowding in the cell or any other sources of stabilization) possibly bringing the conformational transition into the physiological temperature range (Fig. 1). Deletions leading to such an effect, as well as helix-breaking mutations, could introduce flexible links in the chain or at least reduce the Kratky–Porod persistence length of the wormlike coil, which best represents the nature of at least spectrin (Reich et al., 1982; Stokke et al., 1986). This in turn would be expected to change the elastic character of the protein in situ. If our observations on the one repeat are typical of the remainder, then such sequence changes, as much as the length of the dystrophin rod domain in the many known cases in which this element is genetically truncated, may determine the severity of the resulting membrane defect.

We thank J. C. Pinder for help and advice and A. C. M. Harris for determining *N*-terminal sequences. This research was made possible by the support of the Muscular Dystrophy Group.

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