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A proton nuclear magnetic resonance study of the mobile regions of human erythroid spectrin

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Abstract

The effect of added NaCl (0–150 mM) and temperature (6–65°C) on the conformation of erythrocyte spectrin was investigated using 400 MHz ^{1}H NMR. The relatively narrow resonances (20–40 Hz linewidth) in the spectra arising from protons in regions of the molecule undergoing rapid motions were selectively detected using either the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence without water presaturation or a simple $\pi/2$ pulse sequence with water presaturation. The T_2 relaxation of these protons was not influenced by changes in solution conditions (0–150 mM NaCl, 6–37°C) indicating that their motions were independent of the overall shape of the molecule. Significant increases in the areas of the aliphatic peaks for spectrin samples at fixed salt concentrations occurred as the temperature was raised from 6 to 37°C. The increases were independent of the state of polymerization of spectrin and were greater in the absence of added salt above 25°C. The changes reflect increasing numbers of mobile residues, probably due to partial unfolding of spectrin's repeated structural unit. At temperatures above 37°C, sharp increases in the areas of the spectral envelopes reflect cooperative unfolding of spectrin. Comparison with results previously obtained in this laboratory using CD and ORD indicate that at least part of the lost structure is α-helical.

Key words: Spectrin; Nuclear magnetic resonance; Conformational change; Configurational entropy; Erythrocyte; Random coil

1. Introduction

Spectrin is the principal component of the human erythrocyte membrane skeleton, the two-dimensional protein network which underlies the lipid bilayer. The membrane skeleton plays a major role in the membrane's remarkable structural stability and elastic deformability under shear forces imposed by the circulation [1,2]. The individual components of the skeleton and their mode of interaction have been studied extensively [3,4]; however, the mechanical properties of the membrane skeleton are still not understood on a molecular level.

The spectrin heterodimer is composed of an α and β chain, whose cDNA sequences indicate molecular weights of 280 and 246 kDa, respectively [5,6]. 'Head-to-head' association of two het-

Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; FID, free induction decay; pH_{app}, apparent pH; PMSF, phenylmethylsulfonyl fluoride; TSP, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4.

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erodimers forms the tetramer, the main oligomeric form of spectrin in the cytoskeleton [7]. Spectrin heterodimers and tetramers are highly elongated and flexible [8,9], and studies of the molecule's rotational dynamics show that it undergoes segmental motion both in solution and when attached to the membrane [10–12].

Extension and contraction of spectrin has been shown to occur in response to changes in the temperature and ionic strength of the solution [9,13]. In particular, spectrin is more rod-like and stiff at low ionic strength and temperature and progressively more worm-like and compact as the temperature or ionic strength is raised. On the basis of these results, spectrin has been modelled as a polyelectrolyte that behaves as an 'entropic spring'; that is, a molecule whose flexibility is derived from configurational entropy similar to the elasticity of rubber [2,14]. Consistent with this model, the membrane skeleton appears to behave as an ionic gel whose properties have been used to form the basis for predicting red-cell shape and shape change [14].

In spite of the sophistication of these models, they remain highly controversial since several studies indicate that the elastic deformability of spectrin and the erythrocyte depends largely on enthalpic events rather than configurational entropy [15–17]. In particular, Vertessy and Steck [15] have proposed that the elastic deformability of the skeleton is mediated by associations between individual domains within each of spectrin's polypeptide chains.

High-resolution ^{1}H NMR has been used to study selectively the internal motions of very large macromolecules such as myosin [18] and immunoglobulins [19]. Due to the size and relative immobility of these molecules, the protons would be expected to give rise only to extremely broad resonances. However, large proteins can give rise to sharp resonances in the NMR spectrum due to rapid internal motions arising from, for example, hinge regions and regions of random coil. The broad resonances from the relatively immobile protons can be selectively removed using spin-echo techniques. ^{1}H NMR thus provides a sensitive means of investigating the properties of the mobile regions of large proteins.

The ^{1}H NMR spectrum of spectrin also shows several relatively narrow peaks which are estimated to account for 15% of the molecule at 20°C in 150 mM NaCl, pH 7.5 [20–22]. However, the studies to date have not systematically exploited the use of NMR to investigate changes in the flexible regions within spectrin arising from changes in solution conditions.

In the present study, we have used ^{1}H NMR to investigate changes in the size and mobility of the flexible regions of spectrin with changes in the solution conditions. The results show that the number of mobile residues is very sensitive to ionic strength and temperature and suggest that both configurational entropy and enthalpy play a role in the overall flexibility of the spectrin molecule.

2. Materials and methods

2.1. Preparation of spectrin

Spectrin was prepared as previously described [23]. Packed, human red cells were obtained from the Red Cross Blood Bank, Sydney. The packed cells were washed twice in 5 mM sodium phosphate (pH 8) containing 0.95% w/v NaCl and the cells were collected after each wash by centrifugation (10 min, 4000g). The cells were then haemolysed in 5 mM sodium phosphate (pH 8) and the membranes collected by centrifugation at 37000g for 15 min. This procedure was repeated until the membranes were free of haemoglobin.

The membranes were given a final wash in water to lower the ionic strength of the solution. Following centrifugation, the supernatant was removed and the membranes were pooled. PMSF (0.3 mM) was added to the pooled membranes and the membranes incubated at 37°C for 20–60 min to release the spectrin dimer. Vesicles were removed from the incubated sample by centrifugation for 15 min at 27000g, and the supernatant was concentrated by dialysis against Aquacide II (Calbiochem) to 5–10 mL. The concentrated supernatant was clarified by centrifugation (15 min, 27000g).

Spectrin dimer was isolated from the supernatant using repeated gel filtration on a sepharose

CL-4B column (50 cm × 2.5 cm) equilibrated with 10 mM sodium phosphate (pH 7.5), containing 0.1 M NaCl, 5 mM EDTA, 1 mM dithiothreitol and 0.3 mM sodium azide. The concentration of the fractions was determined spectrophotometrically at 280 nm using $E_{1\%,1cm} = 10.7$ [24].

2.2. Preparation of spectrin for NMR studies

Spectrin fractions were pooled and concentrated by vacuum dialysis to 1 mL. The concentration of spectrin ranged from 1–20 mg/mL depending on the preparation. The concentrated sample was dialyzed for 3 h against 3 L of 0.5 mM sodium phosphate with 0.06 mM dithiothreitol and centrifuged at 113000g for 15 min to sediment any insoluble matter. The spectrin was then dialyzed three times against D_2O (99.75%: ANSTO, Lucas Heights, Sydney) containing 0.5 mM sodium phosphate ($pH_{app} = 7.4$ at 20°C). All buffers used following vacuum dialysis were purged with nitrogen to remove dissolved oxygen.

NaCl in deuterated sodium phosphate buffer was added to the concentrated spectrin to give a final concentration in the sample of 1.5 mM phosphate, and either 0, 25, 50, or 150 mM NaCl. The pH_{app} of the samples was 7.5 at 20°C. Chemical shifts were referenced by adding either TSP (0.000 ppm) or dimethyl sulfoxide (2.51 ppm).

2.3. ^1H NMR and processing of spectra

Spectra were acquired using a Bruker AMX 400 MHz wide-bore spectrometer, operating in the pulsed Fourier transform mode. Spectra were collected using a CPMG pulse sequence without water presaturation and a total echo time, τ, of 2 ms. Alternatively, a standard $\pi/2$ pulse sequence was used with or without a 1.0–2.0 s low power presaturation of the HDO resonance. For the CPMG pulse sequence, 16K data points were collected, the spectral width was 7.6 kHz and the total delay between the start of each acquisition was 2.1–5.9 s. For the one-dimensional pulse sequence, 8K data points were collected, the spectral width was 3.8 kHz and the total delay between the start of each acquisition was 2–5.9 s. Two seconds was sufficient for full relaxation, within error, over the range of conditions used for both the CPMG and one-pulse experiments. This was confirmed by measuring T_1 values of the narrow aliphatic peaks using inversion recovery for spectrin at 5°C ± 150 mM NaCl and 37°C ± 150 mM NaCl. For all four conditions, a recycling time of 2 s was equivalent to 4–4.5 T_1; equivalent to > 98% of full recovery.

The CPMG pulse sequence was also used to measure transverse relaxation time (T_2) values. Determination of each value of T_2 involved 16 spin-echo experiments with values of τ ranging from 1.6–120 ms.

The $\pi/2$ and π pulses were measured for each concentration of NaCl used. As expected the length of the pulses increased linearly with increasing salt concentration, being ~ 15% longer in the presence of 150 mM NaCl compared to the value obtained in the absence of NaCl. Temperatures of the sample in the probe were calibrated using either methanol or ethylene glycol [25].

The FIDs were multiplied by a decaying exponential with a line-broadening of 2–3 Hz prior to Fourier transformation. The spectra were plotted using a constant scaling factor and the areas under the spectrin resonances were then integrated numerically using the Bruker software. The areas were adjusted for the effect of salt on the performance on the NMR coil [26] and the effect of temperature on the Boltzmann distribution of the nuclei. Alternatively, the areas of the spectral envelopes were measured relative to the area of TSP. Both methods yielded similar results. In this letter, the spectral envelope for spectrin was limited to the area under the six major aliphatic resonances (labelled 1–6 in Fig. 1b). The resonances to high frequency of 2.4 ppm were not included in the analysis as they were sometimes partially obscured by impurity peaks. The aromatic resonances were also excluded as the signal-to-noise ratio was not always sufficient for precise quantification. The error associated with processing and integration of the spectra was generally ± 5%, determined by repeated processing of one spectrum.

T_2 values were calculated by non-linear least-squares regression of double exponential functions onto plots of peak height versus τ. The

regression program was based on the Marquardt algorithm [27]. Standard errors for the T_2 values were usually much less than 20% of the values themselves.

2.4. Gel electrophoresis

Samples of the purified dimer were examined before and after NMR experiments by SDS-PAGE using a 10% w/v polyacrylamide gel [28]. As observed from previous work on spectrin involving both sedimentation equilibrium and heavily overloaded SDS-PAGE gels [23,29], all preparations were found to be free from contamination and proteolysis products.

The oligomeric state of spectrin was examined by native gel electrophoresis on slab gels containing a linear 2–5% (w/v) polyacrylamide gradient. The buffer included Tris (0.04 M), sodium acetate (0.02 M) and EDTA (2 mM) adjusted to pH 7.4 using acetic acid (modified method of Fairbanks et al. [30]) and was recirculated during electrophoresis to minimize pH changes. SDS was excluded from the gel and the tank buffer. The gels were prerun for 1 h at 50 V at 4°C to remove ammonium persulfate and then the protein samples were electrophoresed for at least 18 h at 50V/4°C.

3. Results

Fig. 1a shows a typical 400 MHz ^{1}H NMR spectrum of spectrin acquired using the $\pi/2$ pulse sequence in the absence of water presaturation. For a structured molecule the size of the spectrin heterodimer ($\sim$ 526 kDa), spin–spin relaxation should be very efficient leading to linewidths of $\sim$ 200–600 Hz [18]. This phenomenon gives rise to the broad envelope of resonances seen in Fig. 1a. Superimposed on the broad envelope are narrow resonances arising from highly mobile protons.

The broad envelope can be removed using spin-echo techniques in which the value of τ is selected to allow relaxation of the resonances from protons in rigid parts of the molecule. Fig. 1b shows the spectrum for a spin-echo experiment in which $\tau = 2$ ms. The broad envelope has been removed thereby enhancing the detection of the narrow resonances arising from the highly mobile regions of the molecule [31]. The pattern of resonances is consistent with the spectra obtained by Fung et al. [21,22] at 200 MHz. although our spectra, obtained at 400 MHz, show a marked improvement in resolution.

The relatively narrow peaks observed in Fig. 1b have linewidths of 20–40 Hz. However, each of the peaks is composed of many overlapping resonances with linewidths of $\sim$ 6 Hz, calculated from the T_2 relaxation times (see below). Comparison of the spectra of native spectrin and spectrin denatured by 4% (w/v) NaOD shows that the linewidths are similar (data not shown), indicating that the narrow resonances arise from disordered regions of the molecule. Consistent with this, the chemical shifts of the peak maxima for both native and denatured spectrin correspond to those expected for residues in random coil [32]. There is no evidence of shifted resonances reflecting the presence of protons in structured parts of the native molecule. For example, there is no evidence of methyl resonances shifted upfield even after resolution enhancement of the spectra (not shown).

The broad envelope of resonances also can be removed by presaturating the HDO resonance. Presaturation not only reduces the size of the HDO peak, but also produces saturation of the backbone CH proton resonances and, through spin diffusion, saturation of all other resonances corresponding to rigid nuclei [33]. The relative sizes of envelope areas obtained with water presaturation over a range of NaCl concentration and temperature (see below) were similar to those obtained using spin-echo techniques, indicating that spin diffusion was not strongly dependent on temperature and salt concentration.

3.1. T_2 relaxation

The effect of ionic strength and temperature on the mobility of the flexible segments of spectrin was investigated by measuring the T_2 values of peaks 1–6 (see Fig. 1b) for spectrin in 0 and 150 mM NaCl, at 6 and 37°C. In a typical experi-

ment, the data could be fitted by a double exponential in which the T_2 values for the fast-decaying components were in the range 1–4 ms and those for the slower-decaying components in the range 20–75 ms, for all conditions examined. The range of T_2 values for the fast and slow decaying components and the independence of T_2 relaxation with respect to changes in salt are very similar to results obtained for myosin [26].

Fig. 2 shows examples of T_2 relaxation for selected resonances at different temperatures and ionic strengths. The data indicate that T_2 relaxation for the individual peaks is independent of the conditions used. A Student's t-test using the method of paired comparisons [34] confirmed that there were no significant changes in T_2 values with either temperature or ionic strength.

3.2. Effect of ionic strength

Fig. 3 shows the dependence of the area of the spectral envelope of spectrin as a function of the NaCl concentration at 25°C. There is a 90% increase in the size of the spectral envelope as

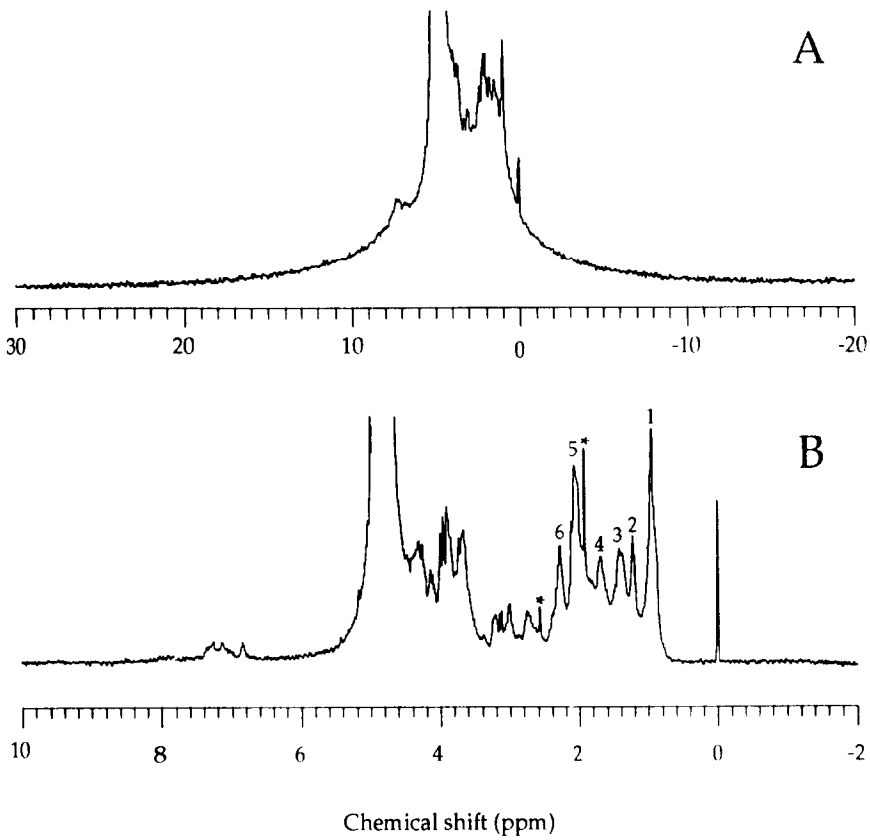

Fig. 1. 400 MHz ^{1}H NMR spectra of spectrin (2.5 mg/mL) in 1.5 mM NaPi, 150 mM NaCl at 25°C, $pH_{app} = 7.5$. (a) Spectrum collected using the $\pi/2$ pulse sequence without presaturation of the HDO resonance. The broad envelope of resonances results from efficient spin–spin relaxation of the protons in folded parts of the molecule. 64 transients were collected and 3 Hz of line-broadening was used prior to Fourier transformation. (b) Spectrum collected using the CPMG pulse sequence. The value of τ (2 ms) was selected to be just sufficient to remove the broad envelope of resonances, thereby enhancing narrow resonances arising from mobile parts of the protein. Peaks labelled 1–6 were used for the measurement of T_2 relaxation times (see text). The asterisks designate resonances from impurities. 128 transients were collected and 2 Hz of line broadening was used.

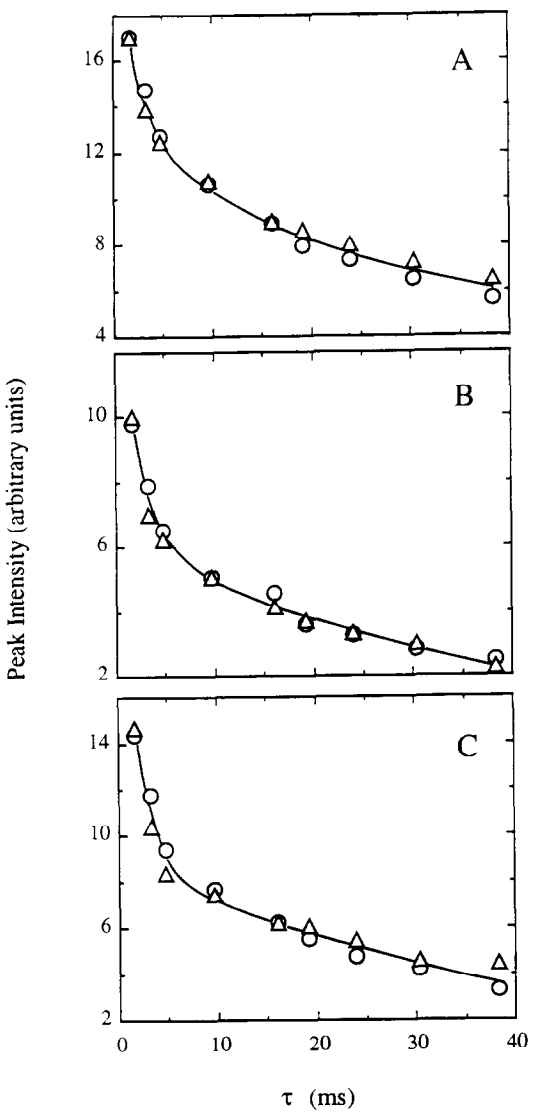

Fig. 2. Typical T_2 relaxations for selected aliphatic peaks at (A) 0.9, (B) 1.4, and (C) 2.1 ppm for spectrin in 150 mM NaCl at 6°C (○), and in the absence of added salt at 37°C (△). The data are plotted as peak intensity versus total echo time, τ. The curves represent the best fits from nonlinear least-squares regression of a double exponential function onto the data. The returned values of T_2 were (A) 2.7 and 55 ms, (B) 1.8 and 37 ms, and (C) 2.0 and 44 ms.

the concentration of NaCl decreases from 150 to 0 mM. Most of the increase occurs between 25 and 0 mM NaCl. Since the T_2 relaxations of the narrow resonances are independent of the conditions used, the increase in the area of the spectral envelope strongly suggests an increase in the number of mobile residues with decreasing salt concentration. Similar results were obtained with KCl.

3.3. Effect of temperature

The effect of temperature on the size of the spectral envelope was examined for spectrin in both 0 and 150 mM NaCl. Fig. 4 shows that there is a significant increase in envelope area between 6 and 37°C. The extent of the increase is markedly dependent on the ionic strength of the sample: below 25°C, there is little difference between the envelope areas for spectrin in 0 and 150 mM NaCl. However, between 25 and 37°C, the envelope area for spectrin in 0 mM NaCl increases by ~250%, compared to ~100% for spectrin in 150 mM NaCl.

The change in envelope area between 6 and 37°C was completely reversible and not dependent on the state of spectrin self-association. This was investigated by comparing spectra taken at 6°C before and after incubating the sample at 37°C for 60 min. As expected, native gel electrophoresis revealed that spectrin kept at 6°C was largely dimeric (35; data not shown). However,

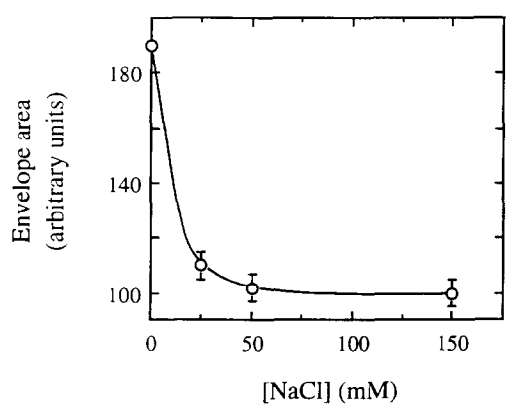

Fig. 3. A typical data set showing the effect of ionic strength on the area of the spectral envelope for spectrin (8.2 g/L) in 1.5 mM NaPi, pH_{app} 7.5, at 25°C. Spectra were collected using the $\pi/2$ pulse sequence with a 1 s presaturation of the HDO resonance. 512 transients were acquired for each spectrum and 2 Hz of line-broadening was used. The error bars indicate uncertainty in processing and integration of the spectra (see section 2).

following incubation at 37°C in 150 mM NaCl, the thermodynamics of the self-association strongly favours the formation of tetramers and higher oligomers [23,29]. Due to a high activation energy, the distribution of oligomers established at 37°C can be largely preserved upon rapid cooling below 20°C [35]. In spite of the large shift in the distribution of oligomers, the spectral envelope areas obtained at 6°C remained identical before and after incubation. Similar reversibility was observed in the absence of added NaCl (data not shown).

Fig. 5 shows the percentage increase in the envelope area between 5 and 65°C for spectrin in 0 mM NaCl and 150 mM NaCl. For both spectra, the most rapid increase in envelope area occurs between 37 and 47°C consistent with co-operative unfolding of secondary structure observed using CD and ORD [36–38]. In 150 mM NaCl, the transition is small, probably due to interactions between residues freed during gross unfolding of secondary structure. This is supported by the fact that at temperatures above 47°C, the samples

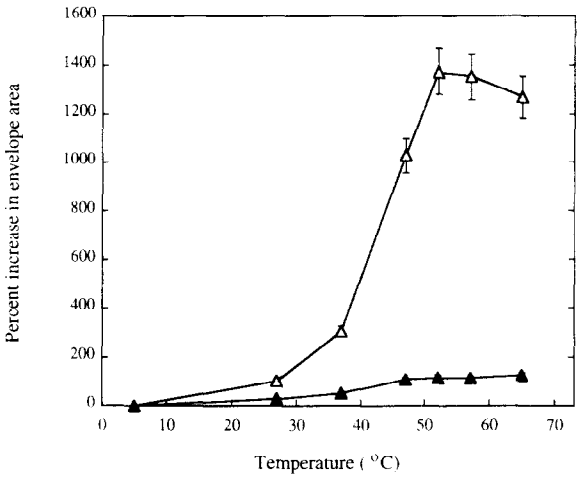

Fig. 5. Changes in envelope area with temperature from 5 to 65°C for spectrin (8 mg/mL) in 1.5 mM NaPi, pH_{app} 7.5, with 0 mM added NaCl (open symbols) and 150 mM added NaCl (solid symbols). Values are plotted as percentage increases with respect to the envelope areas obtained at 5°C. The figure represents a typical set of data. Spectra were acquired using the $\pi/2$ pulse sequence with a 1 s presaturation of the HDO resonance. 512 transients were acquired for each spectrum and 2 Hz of line-broadening was used. The error bars account for uncertainty in processing and integration of the spectra (see section 2).

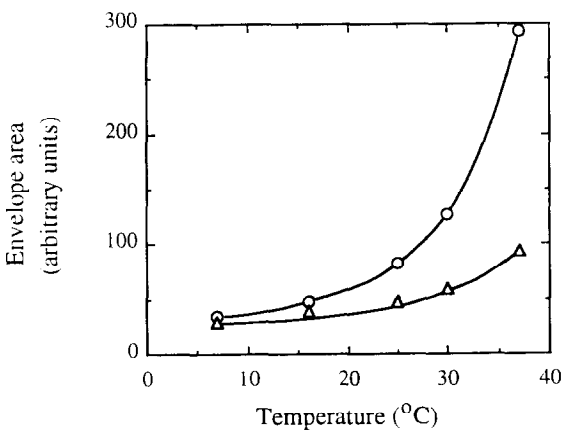

Fig. 4. Effect of temperature between 6 and 37°C on the area of the spectral envelope for spectrin (12 mg/mL) in 1.5 mM NaPi, pH_{app} 7.5, with 0 mM added NaCl (○) and 150 mM NaCl (△). Both samples were made from the same spectrin preparation. The figure represents a typical data set. Spectra were acquired using the CPMG pulse sequence with $\tau = 2$ ms. 128 transients were acquired for each spectrum and 2 Hz of line broadening was used. The error bars are smaller than the symbols in this case (see section 2).

became turbid and there was visible broadening of the NMR spectra, indicating partial immobilization of random structure due to non-specific aggregation. (These phenomena were not observed in the 0 mM NaCl samples.) The mobility of tryptophan residues in native spectrin in 120 mM NaCl decreases above 49°C, again indicating non-specific aggregation of unfolded polypeptide [39].

In 0 mM NaCl, at temperatures above ~ 55°C, there is no change in the envelope areas (Fig. 5). However, comparison of the envelope areas with the sample denatured in 4% w/v NaOD indicated that the sample still had not completely unfolded at 55°C (data not shown). The absence of change above ~ 55°C may be due to non-specific aggregation of the molecule following large scale unfolding, or a lull before another structural transition.

4. Discussion

The α and β subunits of spectrin are composed almost entirely of a 106 amino acid repeat motif (22 and 17 repeats, respectively; 5, 6). Speicher and Marchesi [40] predicted that each repeat unit folds into a triple α-helical bundle with the helices connected by reverse turns. Random coil connects one folded repeat unit with the next giving rise to the extended, worm-like structure of spectrin [8]. It is likely that the rapid internal motions giving rise to the narrow resonances in NMR spectra correspond, a least in part, to these disordered, connecting regions. A previous NMR study of spectrin [22] showed that the narrow resonances represent ~15% of the molecule, in good agreement with the predicted size of these connecting regions [40,41]. It should be stressed, however, that this estimate relates to the specific set of conditions used (20°C, 150 mM NaCl, pH 7.4)

We have observed that the area of the spectral envelope of aliphatic resonances increases with temperature (Figs. 4 and 5) and with decreasing ionic strength (Fig. 3). These increases in envelope area probably represent an increase in random coil in the protein for the following reasons:

(i) T_2 relaxation is not dependent on either salt concentration or temperature. This lack of dependence occurs in spite of the large changes that can occur in the overall shape of the molecule. For example, spectrin adopts relatively compact conformations in the presence of 150 mM NaCl but is fully extended in the absence of added salt [9,13,14]. The fact that T_2 relaxation is independent of overall molecular rotation strongly indicates that larger envelope areas in spin-echo spectra reflect increased numbers of residues involved in rapid internal motions. Increased numbers of random coil residues would be expected to have motions independent of the overall molecular rotation. Similarly, myosin undergoes a salt-dependent increase in the NMR spectral envelope which is independent of T_2 relaxation and has been interpreted as the presence of additional mobile residues [26].

T_2 values in the range 1–4 ms and 20–75 ms correspond to rotational correlation times, τ_c, of $\sim 10^{-7}$ and $\sim 10^{-9}$ s, respectively, based on the rigid-rotor nearest-neighbour model [31]. These estimates of τ_c agree with the two fastest of the three rates of segmental motion ($\tau_c = 10^{-3}$, 10^{-7}–10^{-6} s, and $> 10^{-9}$ s) reported for spectrin using EPR spectroscopy [42]. From the Stokes–Einstein equation, a hydrated sphere with a rotational correlation time of 10^{-9} s would have a radius of ~1 nm, and thus contain around 20 residues (assuming a hydrated specific volume of 1 cm^3 g^{-1}). Our data are therefore consistent with the relaxation of rapid internal motions in spectrin.

(ii) The resonance linewidths of native spectrin are very similar to those obtained for spectrin denatured by hydroxide suggesting that the mobile protons have motional freedom comparable to random coil. Furthermore, there is no evidence in any of the spectra of native spectrin of resonances shifted from random coil positions; such as, for example, methyl resonances shifted upfield from 0.85 ppm. The appearance of narrow, shifted resonances would indicate the presence of at least some highly mobile *ordered* structure such as occurs with stacked bases in single-stranded DNA [31] and the S1 fragment of myosin [18]. In contrast, the homologous fourteenth repeat of the β chain of spectrin (which we have purified from an *Escherichia coli* expression system) clearly shows narrow shifted resonances in the ^{1}H NMR spectrum, including methyl resonances shifted upfield (unpublished data). In the intact spectrin molecule these resonances, which are a reflection of the presence of stable secondary and tertiary structure, are presumably broadened to the point where they cannot be observed in the spin-echo spectra.

(iii) Proteolysis studies are consistent with the presence of random coil in spectrin: Spectrin is susceptible to proteolytic cleavage, particularly in the absence of added salt [43,44]. This susceptibility indicates the presence of exposed, flexible loops, such as occur between the CH1 and CH2 domains of IgG [19]. (The loops in IgG are readily cleaved by papain and pepsin and are the source of the narrow resonances in ^{1}H NMR spectra [19].) Furthermore, the major sites of proteolytic attack in spectrin are known to occur

in the regions *between*, rather than within, the folded repeat units [43,44, unpublished data], consistent with the location of random coil predicted from the amino acid sequence [40].

(iv) Our earlier studies using CD and ORD show that spectrin has less α-helix in 0 mM NaCl compared to 100 mM NaCl at temperatures between 25 and 37°C [36]. Furthermore, the rate of loss of α-helix with increasing temperature is greater in the absence of salt [36]. The loss of helix probably reflects the transition to random coil accompanying the early stages of cooperative unfolding [36].

The trends for the loss of α-helix with temperature, in the presence and absence of added salt, are closely matched by the changes in the NMR spectral envelope areas (Fig. 4). Importantly, since the ratio of α-helix to disordered polypeptide in spectrin is large, the loss of a small proportion of α-helix as measured by CD or ORD is manifested as a relatively large increase in the area of narrow resonances in the NMR spectrum. This demonstrates the usefulness of NMR in monitoring the unfolding of spectrin.

Note that Calvert et al. [20] found no difference in the envelope areas of NMR spectra obtained for spectrin in 5 mM phosphate and 5 mM phosphate plus 150 mM NaCl. However, considering that the ionic strength of 5 mM phosphate is relatively high (~ 12 mM) and that their experiments were performed at 20°C, the difference is, in fact, likely to be negligible (see Figs. 3 and 4).

(vi) The NMR spectral envelopes increase rapidly above 37°C with transitions centred at ≈ 47°C (Fig. 5), closely similar the transition temperature of 49°C for cooperative unfolding [36,37].

In conclusion, both the temperature and ionic strength studies using NMR show the presence of marginally stable secondary structure within spectrin. The increased unfolding at low salt concentrations is probably due to the destabilisation of the α-helix in the absence of charge-screening [45]. On the basis of thermodynamic considerations [46], the loss of secondary structure will occur at the junctions between ordered secondary structure and random coil. Thus, using the model of Speicher and Marchesi [40], α-helix would unfold at the junctions with the random coil segments. This would lead to an increase in the length of the disordered regions connecting the repeat units.

The marked thermal flexing of spectrin even at temperatures between 4 and 38°C [9–11, 47] has been used to model the protein as an entropic spring in which the repeat units are joined by mobile regions [14]. Our results indicate that over the same temperature range the length of the random-coil connecting regions increases. On this basis, the thermal flexing of spectrin will not be due solely to configurational entropy but will include enthalpic components from the folding and unfolding of secondary structure adjacent to the connecting regions. Consistent with this, calorimetry results [36] show a broad enthalpy change for spectrin in the absence of added salt, in keeping with the wide temperature range over which unfolding of the molecule occurs under these conditions (Figs. 4 and 5).

We propose, therefore, that the flexibility of spectrin involves significant contributions from both configurational entropy *and* enthalpy. In contrast, present models propose that spectrin's flexibility is driven almost exclusively by either entropic [14] or enthalpic events [15]. Vertessy and Steck [15] consider that the source of the enthalpic contribution arises from the breaking and reforming of noncovalent associations between the folded repeat units. However, their data could also be interpreted in terms of changes in the unfolding and folding of secondary structure within individual repeat units adjacent to the unfolded connecting regions.

Recently, the crystal structure of a single structural repeat unit of *Drosophila* α-spectrin was reported by Yan et al. [48]. The structure is largely in agreement with the triple-helical-bundle model proposed by Speicher and Marchesi [40] for erythroid spectrin. For *Drosophila* α-spectrin, α-helix extends to the N-terminus and to within two residues of the C-terminus [48]. These terminal regions provide the putative random coil connections between repeat units in erythroid spectrin [40]. It is possible that the residues near the termini in *Drosophila* α-spectrin have a weak predisposition towards forming α-helix which is stabilized in the crystal (a different anomaly in

the crystal structure is discussed by Yan et al. [48]). It is more likely, however, that this apparent discrepancy simply reflects the difference between the uniquely flexible erythroid spectrin and the other, more rod-like, members of the spectrin family. Indeed, our data (unpublished) show that the proteolytically resistant core of the homologous fourteenth repeat of the β-chain of erythroid spectrin (which has the same phasing as the folded repeats of Winograd et al. [44] and Yan et al. [48]) is at least seven residues smaller than that for *Drosophila* α-spectrin repeats: A potential chymotrypsin site near the N-terminus of the erythroid repeat is cleaved. The site is conserved in the *Drosophila* α-spectrin repeats but is not attacked under similar assay conditions [44].

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