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Effect of Ionic Strength on the Organization and Dynamics of Tryptophan Residues in Erythroid Spectrin: A Fluorescence Approach

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Abstract: *The ionic strength of the medium plays an important role in the structure and conformation of erythroid spectrin. The spectrin dimer is a flexible rod at physiological ionic strength. However, lower ionic strength results in elongation and rigidification (stiffening) of spectrin as shown earlier by electron microscopy and hydrodynamic studies. The ionic strength induced structural transition does not involve any specific secondary structural changes. In this article, we have used a combination of fluorescence spectroscopic approaches that include red edge excitation shift (REES), fluorescence quenching, time-resolved fluorescence measurements, and chemical modification of the spectrin tryptophans to assess the environment and dynamics of tryptophan residues of spectrin under different ionic strength conditions. Our results show that while REES, fluorescence anisotropy, lifetime, and chemical modification of spectrin tryptophans remain unaltered in low and high ionic strength conditions, quenching of tryptophan fluorescence by the aqueous quencher acrylamide (but not the hydrophobic quencher trichloroethanol) and resonance energy transfer to a dansyl-labeled fatty acid show differences in tryptophan environment. These results, which report tertiary structural changes in spectrin upon change in ionic strength, are relevant in understanding the molecular details underlying the conformational flexibility of spectrin.* © 2005 Wiley Periodicals, Inc. Biopolymers 77: 325–334, 2005

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INTRODUCTION

Spectrin is the major component of the erythroid membrane skeleton and forms an extensive filamentous intracellular network that acts as a scaffold for cytoplasmic proteins.[1,2] The inherent flexibility of spectrin is an important factor in the elastic deformability of red blood cells, an essential requirement for the passage of these cells in the circulatory system.[3] Several blood diseases are associated with erythrocyte deformation and defects in spectrin. For example, erythrocytes with abnormal shape and reduced deformability lead to various types of hereditary hemolytic anemia that involve mutations in spectrin.[2,4–6] In addition to its primary role in the erythrocyte cytoskeleton, spectrin has been reported to be involved in the maintenance of Golgi structure and function and protein trafficking in early secretory pathways.[7] Interestingly, we have recently shown that erythroid spectrin has chaperone-like activity.[8]

Spectrin is an elongated heterodimer of two subunits α and β (with mol wt of 280 and 246 kDa, respectively). The two subunits are homologous with about a 30% identity and are aligned in the highly elongated, worm-like heterodimer in an antiparallel side-to-side orientation to give a flexible 100-nm rod-shaped molecule with the amino and carboxy termini toward the ends of the rods (see Figure 1). The primary sequence of spectrin is comprised of a series of contiguous motifs called "spectrin repeats" (typically 106 amino acid repeating sequences) that are characteristic of all members of the spectrin family of proteins.[9,10] The spectrin repeats form triple-helical coiled coils connected by helical linkers. These repeats fold independently to form stable structures.[11]

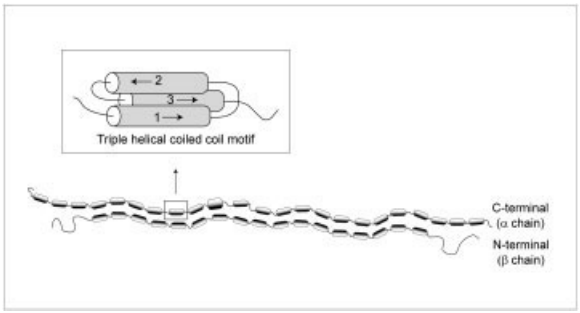

FIGURE 1 A schematic representation of the spectrin dimer. The spectrin heterodimer is formed by the lateral association of the α and β subunits in an antiparallel orientation. The predominant feature of the subunits is the 106 amino acid "spectrin repeat" (shaded cylinders). Each repeat unit ($\sim$23 in α and 17 in β) can independently fold to form a triple-helical coiled coil (see inset). Adapted and modified from Ref. 9.

The ability of spectrin to expand and contract has been attributed to its modular structure made of such repeats.[12]

Studies employing various spectroscopic and physical approaches have shown that spectrin is a highly dynamic protein with multiple classes of internal segmental motions that give rise to its unique organization necessary for its function. Some of the local structural features of spectrin have been shown to be maintained even when denatured by urea.[13] The ionic strength of the medium plays an important role in the structure and conformation of spectrin. Detergent extracted red blood cell membrane skeletons have been shown to expand or shrink with changing ionic strength.[14–16] Electron microscopy studies have shown that at lower than physiological ionic strengths, spectrin appears to be elongated within the membrane skeleton.[17] Interestingly, in studies with the isolated membrane-free spectrin network, the elastic properties of the network were shown to be related to the ionic strength of the medium and the network collapsed due to contraction in high ionic strength environments.[18] The intrinsic viscosity of isolated spectrin heterodimers and heterotetramers show an increase as ionic strength is lowered.[19] In addition, ionic strength was found to increase the cooperativity of unfolding of the spectrin dimer and isolated spectrin repeats.[20] However, despite a significant increase in the Stokes' radius of spectrin at decreased ionic strengths, secondary structural changes in both spectrin dimers and isolated repeats were not detected by differential scanning calorimetry (DSC), CD, and Fourier transform infrared (FTIR) studies.[11,21] It has been postulated that the effect of ionic strength on the hydrodynamic dimensions of spectrin and thermal unfolding could be due to subtle changes in tertiary interactions under such conditions.[20]

The spectrin dimer has a number of tryptophan residues. There are 42 tryptophans in each of the α and β subunits in the spectrin dimer.[22,23] These tryptophans are distributed over the entire spectrin molecule. It is noteworthy that the typically 106 amino acid long repeat units in spectrin have tryptophans strongly conserved at the 45th residue and partially conserved at the 11th residue. Careful examination shows that there are 41 tryptophans in 23 repeat motifs in the α subunit while there are 35 tryptophans in 17 repeat motifs in the β subunit of the spectrin dimer. Importantly, the tryptophans in these positions (repeat motifs) represent more than 90% of the total tryptophans in the spectrin dimer. Some of these conserved tryptophans have been shown to promote folding of spectrin domains[24] and contribute to their thermodynamic stability.[25,26] The fact that tryptophans are distributed over the entire molecule and yet are

localized in the same position in each domain makes them convenient intrinsic fluorescence reporter groups for monitoring conformational changes in spectrin that contribute to its elastic deformability exhibited in physiological conditions.[25] We have recently utilized the intrinsic tryptophan fluorescence of spectrin to detect novel structural features of denatured spectrin[13] and to monitor the interaction of spectrin with micellar detergents[27] and membranes.[28]

Fluorescence spectroscopy offers a powerful approach for monitoring the organization and dynamics of tryptophan residues in proteins due to high sensitivity, suitable time resolution, and multiplicity of measurable parameters.[29,30] In this article, we have used a combination of fluorescence spectroscopic approaches that include red edge excitation shift, fluorescence quenching, resonance energy transfer, time-resolved fluorescence measurements, and chemical modification of the spectrin tryptophans to assess the environment and dynamics of tryptophan residues of spectrin under different ionic strength conditions.

EXPERIMENTAL

Materials

Tris, KCl, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), EDTA, trichloroethanol (TCE), and N-bromosuccinimide (NBS) were from Sigma Chemical Company (St Louis, MO, USA). Ultrapure-grade acrylamide was from Invitrogen Life Technologies (Carlsbad, CA, USA). 11-[(5-dimethylaminonaphthalene-1-sulfonyl]amino)undecanoic acid (DAUDA) was from Molecular Probes (Eugene, OR, USA). The purity of acrylamide was checked from its absorbance using its molar extinction coefficient (ϵ) of $0.23M^{-1}$ cm^{-1} at 295 nm and optical transparency beyond 310 nm.[31] Concentration of stock solutions of DAUDA in methanol were estimated using the molar absorption coefficient (ϵ) of $4{,}800M^{-1}$ cm^{-1} at 335 nm.[32] All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

Isolation and Purification of Spectrin

Clean, white ghosts from goat blood were prepared by hypotonic lysis in 5 m*M* phosphate, 1 m*M* EDTA containing 20 μg/mL of PMSF at pH 8.0 (lysis buffer) following the procedure of Dodge and coworkers.[33] Spectrin dimers were purified as described earlier.[33,34] After washing the membranes thoroughly in lysis buffer, the band 6 depleted ghosts were resuspended in 20 volumes of spectrin removal buffer (0.2 m*M* sodium phosphate, 0.1 m*M* EDTA, 0.2 m*M* DTT, 20 μg/mL PMSF, pH 8.0) and incubated at 37°C for 30 min. Crude spectrin was collected in the supernatant after centrifugation. Spectrin was then purified after concentration by 30% ammonium sulfate precipitation followed by chromatography on Sepharose CL-4B, and stored in a buffer containing 5 m*M* sodium phosphate, 1 m*M* EDTA, 20 m*M* KCl, and 0.2 m*M* DTT, pH 8.0. The purity of the preparation was checked by 7.5% SDS polyacrylamide gel electrophoresis under reducing conditions showing the characteristic bands of spectrin dimer (α-chain of 240 and β-chain of 220 kDa) after Coomassie blue staining. Concentration of spectrin was determined spectrophotometrically using an absorbance of 10.7 at 280 nm for 1% spectrin solution[34] and by Lowry's method.[35] To prepare spectrin at specific ionic strengths, spectrin was dialyzed against 2 L of 10 m*M* sodium phosphate, pH 7.4, with (high ionic strength) or without 150 m*M* NaCl (low ionic strength) for 8 h at 4°C with one change of buffer.

Steady State Fluorescence Measurements

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1-cm pathlength quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. All spectra were recorded using the correct spectrum mode. Background intensities of samples in which spectrin was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ± 1 nm of the ones reported. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation[36]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor, is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples, and average values of anisotropy are given in Table I.

Time-Resolved Fluorescence Measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single-photon counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (16 $\pm$ 1 inches of mercury vacuum) and is run

Table I Fluorescence Characteristics of Spectrin at Different Ionic Strengths

Ionic Strength[a]	Fluorescence Emission Maximum[b] (nm)	Fluorescence Anisotropy[c]	REES (nm)
High	337	0.085 ± 0.002	3
Low	337	0.086 ± 0.001	3

[a] High ionic strength refers to spectrin in 10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4 buffer, while low ionic strength refers to spectrin in 10 m*M* sodium phosphate, pH 7.4 buffer. The concentration of spectrin was 0.6 μM in all cases. See Experimental for other details.

[b] The excitation wavelength was 280 nm.

[c] Calculated using Eq. (1). The anisotropy value represents mean ± standard error of at least three independent measurements. See Experimental for other details. The excitation wavelength was 280 nm and emission was monitored at 337 nm.

at 17–22 kHz. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal to noise ratio, 5000 photon counts were collected in the peak channel. All experiments were performed using excitation and emission slits with a nominal bandpass of 8 nm or less. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam, thereby avoiding any possible photodamage of the fluorophore. The data stored in a multichannel analyzer was routinely transferred to an IBM PC for analysis. Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-\tau/\tau_i) \quad (2)$$

where $F(t)$ is the fluorescence intensity at time t and α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i such that $\Sigma_i \alpha_i = 1$. The decay parameters were recovered using a nonlinear least squares iterative fitting procedure based on the Marquardt algorithm.[37] The program also includes statistical and plotting subroutine packages.[38] The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals,[39] and the autocorrelation function of the weighted residuals.[40] A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.3. Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equation[13]:

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (3)$$

Fluorescence Quenching Measurements

Acrylamide and TCE quenching experiments of tryptophan fluorescence were carried out by measurement of fluorescence intensity after serial addition of small aliquots of either a freshly prepared stock solution of 2*M* acrylamide in water or neat TCE (10.42*M*) to a stirred sample followed by incubation for 3 min in the sample compartment in the dark (shutters closed). The excitation wavelength used was 295 nm and emission was monitored at 337 nm. The fluorescence intensities obtained were corrected for dilution. Corrections for inner filter effect for acrylamide quenching were made using the following equation[13]

$$F = F_{obs}\,\text{antilog}[(A_{ex} + A_{em})/2] \quad (4)$$

where F is the corrected fluorescence intensity and F_{obs} is the background subtracted fluorescence intensity of the sample (also corrected for dilution). A_{ex} and A_{em} are the measured absorbance at the excitation and emission wavelengths. The

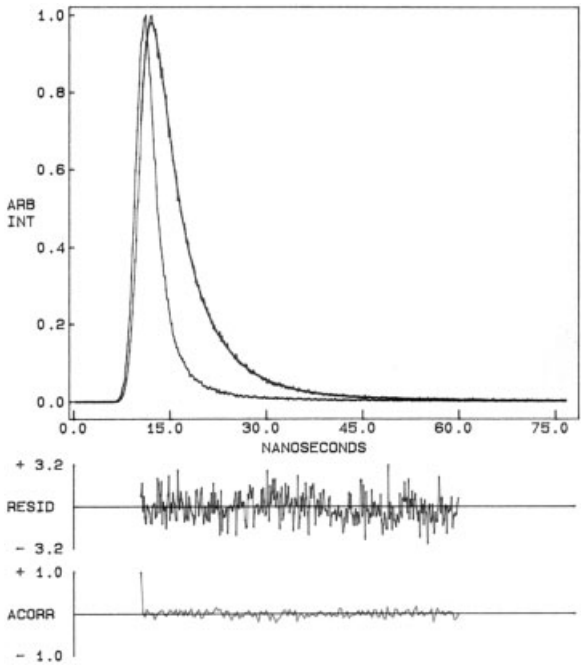

FIGURE 2 Time-resolved fluorescence intensity decay of spectrin at high ionic strength. Excitation wavelength was 297 nm, which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 340 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, with its biexponential fitting. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. The concentration of spectrin was 0.6 μM and the buffer used was 10 m*M* phosphate, 150 m*M* NaCl, pH 7.4. See Experimental for other details.

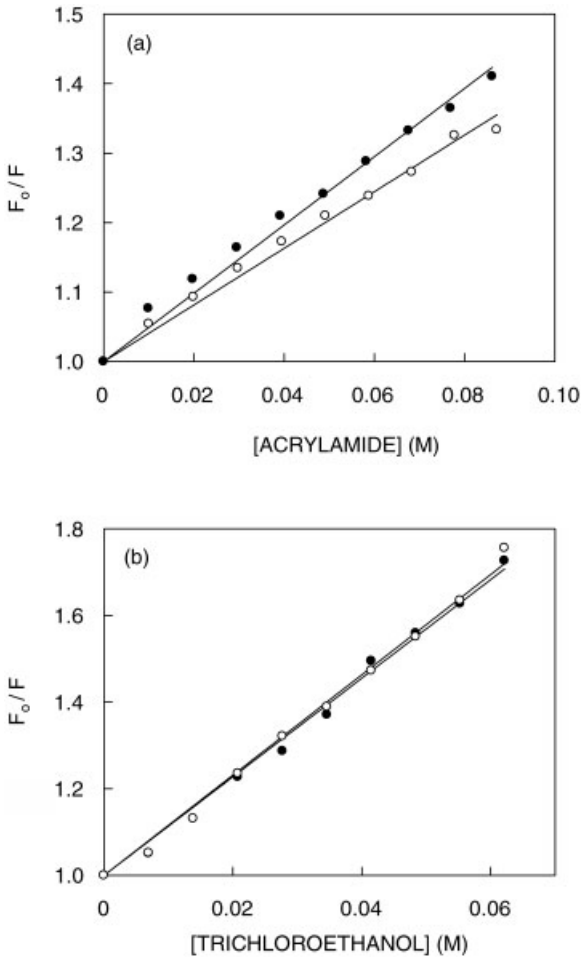

FIGURE 3 Quenching of spectrin tryptophan fluorescence using quenchers of varying degrees of accessibility. Representative data for Stern–Volmer analysis of (a) acrylamide and (b) TCE quenching of spectrin fluorescence at low ionic strength buffer (10 m*M* phosphate, pH 7.4) (○), and high ionic strength buffer (10 m*M* phosphate, 150 m*M* NaCl, pH 7.4) (●). F_o is the fluorescence in the absence of quencher, F is the corrected fluorescence in the presence of quencher. The excitation wavelength was fixed at 295 nm and emission was monitored at 337 nm. The concentration of spectrin used was 0.1 μM. See Experimental for other details.

absorbancies of the samples were measured using a Hitachi U-2000 UV-visible absorption spectrophotometer. Inner filter effects in case of TCE quenching were negligible. Quenching data were analyzed according to the Stern–Volmer equation[13]:

$$F_o/F = 1 + K_{SV}[Q] = 1 + k_q\tau_o[Q] \quad (5)$$

where F_o and F are the fluorescence intensities in the absence and presence of the quencher (acrylamide or TCE), respectively, $[Q]$ is the molar quencher (acrylamide or TCE) concentration, and K_{SV} is the Stern–Volmer quenching constant. The Stern–Volmer quenching constant K_{SV} is equal to $k_q\tau_o$ where k_q is the bimolecular quenching constant and τ_o is the lifetime of the fluorophore in the absence of quencher.

NBS Modification

Spectrin was subjected to NBS modification by serial addition of small aliquots from a freshly prepared 2 m*M* stock solution of NBS in water to a stirred solution of the sample. The sample was incubated in the sample compartment of the fluorimeter in the dark (shutters closed) for 3 min prior to measurement of fluorescence intensity. The background-subtracted fluorescence intensity was corrected for dilution. The excitation wavelength used was 295 nm and emission was monitored at 337 nm. The fluorescence intensity of spectrin in the absence of NBS served as a control. All experiments were done with multiple sets of samples, and average values are shown in Figure 4.

Fluorescence Resonance Energy Transfer Experiments

Fluorescence resonance energy transfer experiments using spectrin tryptophans as donor and DAUDA as acceptor were carried out by measurement of tryptophan fluorescence intensity after serial addition of small aliquots of DAUDA in methanol (3.8 m*M*) to a stirred sample followed by incubation for 30 min in the sample compartment in the dark (shutters closed). The excitation wavelength used was 295 nm and emission was monitored at 337 nm. Energy transfer efficiencies were calculated using the equation[36]:

$$E = (1 - F/F_o) \quad (6)$$

where E is the efficiency of energy transfer, and F and F_o are fluorescence intensities of the donor (spectrin tryptophans) in the presence and absence of the acceptor (DAUDA), respectively.

RESULTS

Table I shows that the maximum of fluorescence emission* of spectrin is at 337 nm, at both low and high ionic strength conditions. The absence of any shift in the fluorescence emission maximum indicates that it is not sensitive to the ionic strength of the medium. The steady state fluorescence anisotropy of spectrin tryptophans is shown in Table I. The anisotropy values indicate that the spectrin tryptophans, on average, are in a motionally restricted environ-

* We have used the term *maximum of fluorescence emission* in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the center of mass of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.

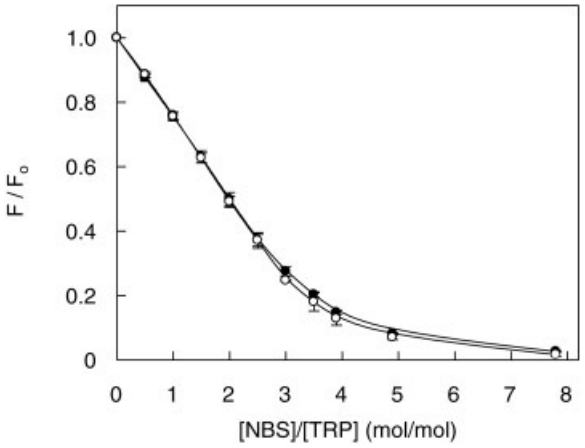

FIGURE 4 NBS modification of spectrin tryptophan residues. Residual fluorescence of spectrin after NBS modification plotted as a function of molar ratio of NBS to spectrin tryptophans at low (○) and high ionic strength (●). F_o is the fluorescence in absence of NBS and F is the fluorescence in presence of increasing concentrations of NBS. The excitation wavelength was fixed at 295 nm and emission was monitored at 337 nm. Data shown represent the mean ± standard error of multiple measurements. All other conditions are as in Figure 3. See Experimental for other details.

ment.[13,41] However, the anisotropy values for the tryptophans appear to be similar in both conditions of ionic strength. This could imply that the average rotational mobility of the spectrin tryptophans is more or less similar in low and high ionic strength conditions, especially keeping in mind the corresponding values for fluorescence lifetime (see later, Table II). Interestingly, these anisotropy values also indicate a lack of any significant energy transfer among tryptophans, although spectrin has a number of tryptophan residues. The relatively large Stokes' shift (~50–60 nm) displayed by spectrin tryptophans could also account for this.[29]

REES represents a powerful approach that can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system, such as proteins or membranes.[42] A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed REES. This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime.[42] The unique feature of REES is that while all other fluorescence techniques (such as fluorescence quenching, energy transfer, and anisotropy measurements) yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics, which is not possible to obtain by other techniques. We have previously shown that REES and related techniques (wavelength-selective fluorescence approach) serve as powerful tools to analyze the conformation and dynamics of tryptophan residues in denatured spectrin,[13] the cytoskeletal protein tubulin,[41] and the soluble hemolytic protein α-toxin.[43]

The magnitude of REES of the spectrin tryptophan residues at low and high ionic strengths is shown in Table I. As the excitation wavelength is changed from 280 to 307 nm, the maximum of emission wavelength of the spectrin tryptophans is shifted from 337 to 340 nm in both cases. The magnitude of REES corresponds to 3 nm irrespective of the ionic strength. Such dependence of the emission maximum on excitation wavelength is characteristic of REES. This implies that tryptophans in dimeric spectrin in these conditions are localized in a motionally restricted environment. Spectrin is a multitryptophan protein and therefore the red edge shift may be indicative of the average environment experienced by the tryptophans. Nevertheless, such a result would directly imply that the regions surrounding at least some of the spectrin tryptophans offer considerable restriction to the reorientational motion of the solvent (water) dipoles around the excited state tryptophans. This is significant since some of the functionally important spectrin tryptophans are localized in the invariant region and are shielded from the bulk (characterized by fast solvent reorientational motion) solvent.[24,26] In addition, many of these tryptophans are at or in the vicinity of hydrophobic patches in spectrin that can bind hydrophobic ligands.[44] Based on binding of the hydrophobic fluorescent probe pyrene, the apparent dielectric constant of the hydrophobic pyrene binding site in spectrin has been estimated to be ~7.[45] The low dielectric characteristics coupled with the presence of restricted water molecules contribute in making some

Table II Fluorescence Lifetimes of Spectrin Tryptophans at Different Ionic Strengths[a]

Ionic Strength	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle^b$ (ns)
High	0.27	5.83	0.73	2.06	3.99
Low	0.28	5.55	0.72	1.63	3.86

[a] The excitation wavelength was 297 nm and emission was monitored at 340 nm. All other conditions are as in Table I. See Experimental for other details.

[b] Calculated using Eq. (3)

of these regions ideal environments for exhibiting REES and related effects.[42]

The dynamic properties of the protein matrix surrounding a given amino acid residue or fluorophore either covalently attached or partitioned from the aqueous phase can be examined from the rate at which this matrix responds to (or relaxes around) the newly created excited state dipole moment of the fluorophore.[46] In other words, the magnitude of REES can be utilized to estimate the relative rigidity of the region of the protein surrounding the fluorophore. For example, the tryptophans in the cytoskeletal protein tubulin exhibit REES of 7 nm in its native state due to the motional restriction experienced by the tryptophans in their immediate environment.[41] The magnitude of REES, however, decreases at high temperatures, and upon denaturation with 8*M* urea, no REES is observed, suggesting a drastic change of the tryptophan environment in the protein matrix and increased rates of solvent reorientation.[41] The invariance of the magnitude of REES with ionic strength in this case would imply that the average motional restriction experienced by spectrin tryptophans is not dependent on the ionic strength of the medium. Therefore, changing ionic strength has no effect on the relative rigidity of the protein matrix around the tryptophan residues.

Fluorescence lifetime serves as a sensitive indicator for the local environment in which a given fluorophore is placed.[47] A typical decay profile of spectrin tryptophans with its biexponential fitting and the statistical parameters used to check the goodness of the fit is shown in Figure 2. The fluorescence lifetimes for spectrin tryptophans at different ionic strengths are shown in Table II. All fluorescence decays could be fitted to a biexponential function using the criteria of satisfying the statistical parameters within a range of values. The mean fluorescence lifetimes were calculated using Eq. (3) and are shown in Table II. The mean fluorescence lifetime of spectrin tryptophans does not differ significantly under these conditions of ionic strength and is ~3.9 ns. This could be attributed to the averaging effect, which does not allow us to pinpoint any possible difference in individual tryptophan lifetimes.

Previous work has shown that ionic strength induced contraction and expansion of spectrin could not be explained on the basis of secondary structural changes either in intact spectrin[21] or in isolated spectrin repeat sequences.[20] Our results utilizing REES, fluorescence anisotropy, and time-resolved fluorescence measurements show no appreciable difference in the environmental dynamics of spectrin tryptophans due to varying ionic strength conditions. This may suggest that there are no distinct alterations in the overall environment of tryptophan residues due to possible rearrangements of helices in the triple-helical repeat regions, where more than 90% of tryptophan residues in the spectrin dimer are located.[13] We chose to further monitor any possible changes in tryptophan environments due to changes in ionic strength by assessing the accessibility of tryptophan residues to aqueous neutral quenchers.

Table III Quenching of Tryptophan Fluorescence of Spectrin at Different Ionic Strengths Using Various Quenchers

Quencher Used	Ionic Strength	K_{sv}[a] (M^{-1})	k_q ($\times 10^{-9}$)[b] (M^{-1} s^{-1})
Acrylamide	High	5.03 ± 0.10	1.26
	Low	4.22 ± 0.18	1.09
TCE	High	13.62 ± 0.41	3.41
	Low	13.56 ± 0.55	3.51

[a] Calculated using Eq. (5). The quenching parameter represents mean ± standard error of at least five independent measurements while quenching data shown in Figure 3 are from representative experiments. See Experimental for other details.

[b] Calculated using mean fluorescence lifetimes from Table II and using Eq. (5).

Acrylamide quenching of tryptophan fluorescence is widely used to monitor tryptophan environments in proteins and peptides.[48] Figure 3a shows representative Stern–Volmer plots of acrylamide quenching of spectrin tryptophans at different ionic strengths. The slope (K_{SV}) of such a plot is related to the accessibility (degree of exposure) of the tryptophans to the aqueous quencher. The linearity of the plot with no apparent downward curvature indicates minimal heterogeneity in tryptophan population as monitored by acrylamide in this concentration range. The quenching parameters obtained by analysis of Stern–Volmer plots are shown in Table III. The Stern–Volmer constant (K_{SV}) for acrylamide quenching of spectrin at low ionic strength was found to be $4.22M^{-1}$. The value of K_{SV} for spectrin in the high ionic strength conditions, on the other hand, was found to be higher ($5.03M^{-1}$), indicating increased exposure of the tryptophans at high ionic strength condition. The K_{SV} for acrylamide quenching of spectrin tryptophans therefore increases by ~19% with an increase in ionic strength. The bimolecular quenching constants (k_q) for acrylamide quenching are also shown in Table III. The k_q values are in overall agreement with Stern–Volmer constants, implying increased accessibility of acrylamide to spectrin tryptophans at higher ionic strength.

TCE is a hydrophobic neutral quencher of tryptophan fluorescence that is less polar than acrylamide

and has been earlier shown to be a more effective hydrophobic quencher than acrylamide.[43,49] The advantage of TCE as a quencher lies in the fact that it can penetrate into the interior of the protein matrix due to its relatively nonpolar nature. The representative Stern–Volmer plots of TCE quenching of spectrin tryptophans at different ionic strengths are shown in Figure 3b. The Stern–Volmer plots obtained at either ionic strength are linear. The quenching parameters (K_{SV} and k_q) obtained by analysis of Stern–Volmer plots are shown in Table III. The increased K_{SV} for TCE quenching ($\sim 13.6 M^{-1}$) of spectrin tryptophans as compared to quenching by acrylamide points to the presence of hydrophobic patches[44] of low polarity[45] in the vicinity of tryptophan residues since TCE is known to preferentially quench tryptophans localized in such areas.[49] Interestingly, Table III shows that there is no significant difference between the values of K_{SV} (or k_q) obtained for spectrin at different ionic strengths. This implies that the accessibility of tryptophan to TCE remains invariant with the ionic strength of the medium. Thus, while spectrin tryptophans are more accessible to acrylamide at higher ionic strengths, accessibility to the hydrophobic quencher TCE is independent of ionic strength. The ionic strength induced conformational changes in spectrin are therefore characterized by changes in overall accessibility of the exposed tryptophan residues while keeping the accessibility of the core tryptophans invariant. Further analysis in terms of contribution of individual tryptophan residues is complicated due to the heterogeneity in fluorescence parameters (such as quantum yield and lifetime) of individual tryptophans in multitryptophan proteins such as spectrin because of environmental sensitivity.[30]

The accessibility of the spectrin tryptophans to aqueous agents was further explored by chemical modification using NBS as an oxidant. NBS oxidation of the indole moiety of tryptophan to the nonfluorescent oxindole[50] can be used to assess the relative accessibility of tryptophan residues in proteins and peptides.[43,51–53] Figure 4 shows the residual fluorescence intensity of spectrin at different ionic strengths after NBS modification of tryptophans plotted as a function of molar ratio of NBS to spectrin tryptophan content. It is apparent from the figure that the sensitivity of spectrin tryptophans to NBS modification is similar at both low and high ionic strengths. For example, at a molar ratio of NBS to tryptophan of ~ 2, fluorescence intensity was reduced to 50% at either ionic strength. Although NBS has previously been shown to be inefficient at modifying deeply buried tryptophan residues,[43,51] Figure 4 shows that spectrin tryptophans are completely accessible to modification by NBS, resulting in less than 10% of original fluorescence at molar ratios of NBS to tryptophan of 5 or higher.

As mentioned earlier, many of the tryptophan residues of spectrin are at or in the vicinity of hydrophobic patches, which can bind hydrophobic ligands such as fatty acids and phospholipids.[44] It has been shown that the binding of ligands at such hydrophobic patches can quench spectrin tryptophan fluorescence.[54] We used a dansyl-labeled fatty acid (DAUDA) to probe any possible alterations in the relative interactions of such hydrophobic patches and spectrin tryptophan residues under conditions of altered ionic strength. The dansyl group is an extensively used extrinsic fluorescent moiety of proteins that can act as an acceptor of tryptophan fluorescence.[36] The Förster distance (R_o), defined as the distance at which the energy transfer efficiency is 50%, for a tryptophan–dansyl pair, has been earlier estimated to be $\sim$21–24 Å.[55] In contrast, the quenching of tryptophan fluorescence by aqueous quenchers such as acrylamide and TCE is considerably more short range and requires molecular contact.[30] Thus, energy transfer of spectrin tryptophans to dansyl-labeled fatty acids serves as a convenient tool to monitor relatively long-range and more global changes in spectrin conformation. Figure 5 shows the energy transfer efficiency as monitored by quenching of spectrin tryptophan (donor) fluorescence as a function of DAUDA concentration at low and high ionic

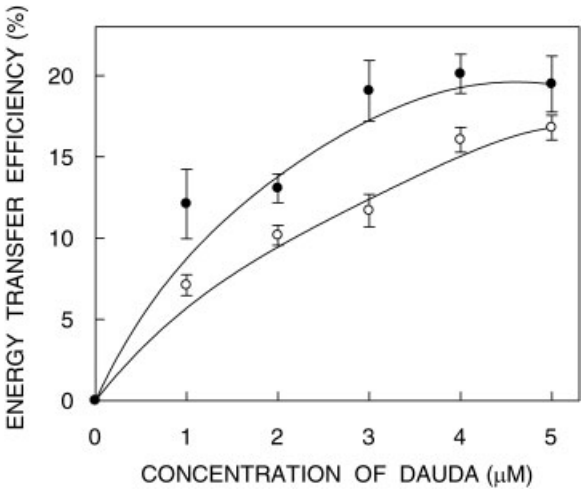

FIGURE 5 Fluorescence resonance energy transfer of spectrin tryptophan fluorescence. Efficiency of energy transfer from spectrin tryptophan to DAUDA as a function of DAUDA concentration at low (○) and high ionic strength (●). The excitation wavelength was fixed at 295 nm and emission was monitored at 337 nm. Data shown represent the mean ± standard error of at least three independent measurements. All other conditions are as in Figure 3. See Experimental for other details.

strengths. Interestingly, the energy transfer efficiencies are found to be greater in high ionic strength conditions at all concentrations of DAUDA used. We attribute the relatively higher energy transfer efficiency in high ionic strength conditions to the increased exposure of hydrophobic areas in the vicinity of tryptophan residues under such conditions.

CONCLUSION

Previous electron microscopy and hydrodynamic data have shown that although the spectrin dimer is flexible at physiological ionic strength, lower ionic strength results in elongation and rigidification (stiffening) of the spectrin dimer in situ (as part of the membrane skeleton) and in vitro (for isolated dimers).[17,19–21] In addition, ionic strength has also been observed to have subtle effects on the thermal unfolding of spectrin peptides consisting of the triple-helical domain.[20] However, such an ionic strength induced transition does not involve any specific secondary structural changes as monitored by CD and FTIR spectroscopy.[20,21] The structural changes in conditions of low ionic strength are thought to be subtle changes that occur near the interdomain regions of the protein.

Since fluorescence spectroscopy is ideally suited to monitor local structural changes and spectrin has appropriately placed tryptophan residues, we used a variety of fluorescence approaches to probe subtle structural changes that accompany the elongation and rigidification observed at low ionic strength. Our results show that any conformational change that may be implicated in the expansion of spectrin dimers with reduced ionic strength does not involve changes in the dynamics and local environment of the tryptophan residues as monitored by REES, fluorescence anisotropy, and lifetime measurements. However, quenching of tryptophan fluorescence by the aqueous quencher acrylamide (but not the hydrophobic quencher TCE) indicates differences in tryptophan accessibility to the aqueous environment. In addition, energy transfer experiments with a dansyl-labeled fatty acid point to possible alterations in the hydrophobic binding sites in the vicinity of tryptophan residues with ionic strength. The ionic strength induced conformational changes in spectrin appear to be characterized by changes in overall accessibility of the exposed tryptophan residues while keeping the accessibility of the core tryptophans invariant. These results, which report tertiary structural changes in spectrin upon change in ionic strength, are relevant in understanding the molecular details underlying the conformational flexibility of spectrin.

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