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REVIEW PAPER

Spectrin Organization and Dynamics: New Insights

Abhijit Chakrabarti · Devaki A. Kelkar · Amitabha Chattopadhyay

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Abstract Spectrin is the major constituent protein of the erythrocyte cytoskeleton which forms a filamentous network on the cytoplasmic face of the membrane by providing a scaffold for a variety of proteins. In this review, several aspects of spectrin organization are highlighted, particularly with respect to its ability to bind hydrophobic ligands and its interaction with membrane surfaces. The characteristic binding of the fluorescent hydrophobic probes Prodan and pyrene to spectrin, which allows an estimation of the polarity of the hydrophobic probe binding site, is illustrated. In addition, the contribution of uniquely localized and conserved tryptophan residues in the 'spectrin repeats' in these processes is discussed. A functional implication of the presence of hydrophobic binding sites in spectrin is its recently discovered chaperone-like activity. Interestingly, spectrin exhibits residual structural integrity even after denaturation which could be considered as a hallmark of cytoskeletal proteins. Future research could provide useful information about the possible role played by spectrin in cellular physiology in healthy and diseased states.

Keywords Spectrin · Hydrophobic binding site · Prodan · Membrane · Chaperone · Tryptophan · REES

Abbreviations

2,3-DPG	2,3-Diphosphoglycerate
ANS	1-Anilinonapthalene-8-sulfonic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine

A. Chakrabarti (✉)
Biophysics Division, Saha Institute of Nuclear Physics, Kolkata 700 064, India
e-mail: abhijit.chakrabarti@saha.ac.in

D. A. Kelkar · A. Chattopadhyay (✉)
Centre for Cellular and Molecular Biology, Hyderabad 500 007, India
e-mail: amit@ccmb.res.in

PRODAN 6-Propionyl-2-dimethylaminonaphthalene
REES Red edge excitation shift

Introduction

Spectrin is the major constituent protein of the erythroid membrane skeleton and forms an extensive filamentous intracellular network that acts as a scaffold for cytoplasmic proteins (Bennett and Gilligan 1993; Winkelmann and Forget 1993). Spectrin was first isolated as a membrane-bound protein from erythrocyte ghosts (hence the name spectrin, which means derived from ghosts in Latin) that was capable of forming coiled filaments visible in electron microscopy (Marchesi and Steers 1968). In order to establish the extensive two-dimensional intracellular network, spectrin interacts with a large number of proteins such as actin, ankyrin, adducin and band 4.1 (Hartwig 1994, 1995). Interestingly, the hemoglobin binding properties of spectrin have recently been established (Chakrabarti et al. 2001; Datta et al. 2003). The interaction of the spectrin-based protein network with the cytoplasmic surface of the membrane controls the elasticity of the bilayer membrane and erythrocyte shape. The inherent flexibility of spectrin is believed to be a major factor that contributes to the elastic deformability displayed by red cells during their passage through the circulatory system (Elgsaeter et al. 1986). Several blood diseases are associated with erythrocyte deformation and defects in spectrin. For example, mutations in spectrin that result in erythrocytes with abnormal shape and reduced deformability lead to various types of hereditary hemolytic anemia (Winkelmann and Forget 1993; Delaunay and Dhermy 1993; Wichterle et al. 1996; Gallagher et al. 1997). 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 (De Matteis and Morrow 2000) and endocytosis (Phillips and Thomas 2006). A spectrin-based network has been implicated as a membrane protein-sorting machine (Beck and Nelson 1996).

Spectrin is a large dimeric amphiphilic protein and is an elongated heterodimer of two subunits α and β (with mol. wts. of 280 and 246 kDa, respectively), with a modular structure made up of typically 106 amino acid contiguous motifs called 'spectrin repeats' (Speicher and Marchesi 1984). It belongs to a superfamily of F-actin crosslinking proteins that includes proteins such as dystrophin and α-actinin, which contain arrays of such 'spectrin repeat' motifs and are thought to have arisen by gene duplication (Thomas et al. 1997; Viel 1999). The spectrin repeats form triple helical coiled coils connected by helical linkers. The ability of spectrin to expand and contract (Shotton et al. 1979) has been attributed to its modular structure made of such repeats (Grum et al. 1999). Besides these features, spectrin exhibits additional structural motifs. These include an actin-binding domain, a pleckstrin homology (PH) domain, a Src homology 3 (SH3) domain, and a calmodulin-like domain. These structural features allow spectrin to take part in a number of physiological events through protein–protein interactions.

The two subunits (α and β) are homologous with about 30% identity and are aligned in the highly elongated, worm-like heterodimer in an antiparallel side-to-side orientation to form a flexible 100 nm rod-shaped molecule with the amino and carboxy termini toward the ends of the rods (see Fig. 1). These heterodimers associate head-to-head to

form 200 nm tetramers and higher order oligomers. In order to form the tetramer, the amino terminal region of the α chain of one dimer is joined with the carboxy terminal end of the β chain of the other dimer, generating a triple helical domain known as the self-association domain (Ralston 1991; Begg et al. 1997). The N-terminal end of the α-subunit begins with an isolated C′ helix (α_1) of 30 residues whereas the β-subunit ends in an incomplete 17th repeat (β_{17}) of 60 residues, consisting of helices A′ and B′, followed by an unfolded segment called domain III. To form the biologically relevant tetramer, helices A′ and B′ interact with helix C′ with low affinity (K_d values are in the μM range). The triple helical structure thus generated is similar to the repeats along the remainder of the spectrin molecule (Ralston 1991; Hartwig 1995; Begg et al. 1997).

The structure of the triple helical repeat domain of spectrin has been solved by solution NMR of the 16th repeat from chicken brain α-spectrin (Pascual et al. 1997). The triple helical repeat was shown to be a left handed coiled-coil, composed of three long helices (A, B, and C) separated by a loop and turn region which are comparatively more mobile in the NMR time scale. Interestingly, the spectrin repeat motifs (106 amino acid) do not form completely independent domains. While an expressed peptide composed of a single motif was found to form a compact, proteolytically resistant unit, peptides composed of two or three motifs in tandem were shown to be substantially more stable (Menhart et al. 1996). Such interactive stabilization of spectrin repeats could either be a result of the difficulty in predicting motif boundaries (DeSilva et al. 1997) or a result of cooperativity of unfolding of tandem repeats (Batey et al. 2005; An et al. 2006). In light of the fact that spectrin repeats may not always behave as classical independent folding units (Batey et al. 2005; An et al. 2006), and the role of the linker regions in spectrin flexibility (a key feature of its cellular role) (Grum et al. 1999; Kusunoki et al. 2004), it would be prudent to examine the organization and dynamics of spectrin in its intact state rather than at the level of individual repeat sequences. The focus of this review is to highlight the organization and interactions of the spectrin tetramer as a whole rather than at the level of specific repeat sequences.

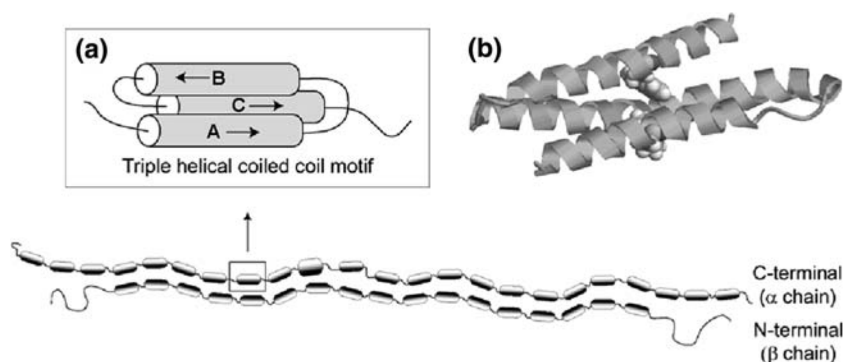

Fig. 1 A schematic representation of the spectrin dimer. The spectrin heterodimer is formed by the lateral association of the α and β subunits in an antiparallel orientation. The predominant feature of the subunits is the 106 amino acid 'spectrin repeat' (shaded cylinders). Each repeat unit (~23 in α and 17 in β) can independently fold to form a triple helical coiled coil (see insets **a** and **b**). Adapted and modified from Kelkar et al. (2005). Inset (**b**) shows a representation of the solution NMR structure of the spectrin repeat (Pascual et al. 1997) with the tryptophan residues highlighted, drawn using RASMOL ver. 2.7.2.1 (Sayle and Milner-White 1995) and coordinates from (PDB code 1AJ3)

Interaction of Hydrophobic Probes with Spectrin: The Binding of Prodan and Pyrene to Spectrin

Spectrin is amphiphilic in nature and is characterized by hydrophobic stretches in its polypeptide sequence containing a large number of hydrophobic sites which can bind hydrophobic molecules, fatty acids, detergents, and phospholipids (Isenberg et al. 1981; Cohen et al. 1986; Streichman et al. 1991; Kahana et al. 1992; Subbarao and MacDonald 1994; Diakowski and Sikorski 1995, An et al. 2004; Ray and Chakrabarti 2003, 2004). The hydrophobic binding sites in spectrin are crucial since this region is believed to facilitate the interaction of spectrin with membranes (see later). Interestingly, many of these sites contain or are close to tryptophan residues in spectrin (see later). The interaction of spectrin with hydrophobic ligands can therefore be conveniently monitored utilizing fluorescence-based approaches (Isenberg et al. 1981; Sikorski et al. 1987; Kahana et al. 1992; Subbarao and MacDonald 1994; Ray and Chakrabarti 2003, 2004). In addition, the binding of unrelated hydrophobic ligands such as hemin, protoporphyrin, DNA-binding drugs and local anesthetics to spectrin has been reported (Beaven and Gratzer 1978; Cassoly 1978; Majee and Chakrabarti 1995; Majee et al. 1999; Mondal and Chakrabarti 2002). Table 1 shows representative binding characteristics for the binding of selected hydrophobic ligands to erythroid spectrin.

Prodan (6-propionyl-2-dimethylaminonaphthalene) is a fluorescent hydrophobic probe whose binding to spectrin has been particularly useful (Chakrabarti 1996; Bhattacharyya et al. 2004). Prodan, introduced by Weber and Farris (1979), is an extrinsic fluorescent probe which has been shown to bind hydrophobic sites of proteins (Weber and Farris 1979; Mazumdar et al. 1992). It is a naphthalene derivative characterized by both electron donor and electron acceptor substituents resulting in a large change in dipole moment upon excitation (Balter et al. 1988; Samanta and Fessenden 2000), leading to extensive solvent polarity dependent shifts in the fluorescence emission maximum. Prodan and its derivatives have earlier been successfully used as hydrophobic markers to estimate the polarity of the heme-binding pocket in apomyoglobin (Macgregor and Weber 1986). However, Prodan could not be effectively used to probe hydrophobic pockets of proteins due to its relatively weak binding to proteins. For example, the affinity of Prodan binding is modest in cases of binding to bovine serum albumin and tubulin (dissociation constants of 1×10^{-5} M and 2×10^{-5} M, respectively) (Weber and Farris 1979; Mazumdar et al. 1992). On the other hand, Prodan was found to bind erythroid spectrin with high affinity (characterized by a dissociation constant of 0.5×10^{-6} M, see Table 1). Importantly, the affinity of Prodan for binding to spectrin is higher by an order of magnitude from the affinities reported previously for binding to other proteins (Chakrabarti 1996).

Table 1 Binding of hydrophobic ligands to erythroid spectrin

Hydrophobic ligand	Dissociation constant, K_d (M)	Stoichiometry
ATP[a]	1.1×10^{-3}	50–100
2,3-DPG[b]	1.1×10^{-3}	50–100
Dibucaine[c]	3.5×10^{-5}	200
Ethidium bromide[d]	1.3×10^{-5}	45–50
Chromomycin[d]	5×10^{-6}	45–50
Mithramycin[d]	2.6×10^{-6}	45–50
ANS[e]	2.5×10^{-5}	4
Prodan[e,f]	0.5×10^{-6}	1
Pyrene[g]	1.6×10^{-7}	1

[a] Chakrabarti et al. (2001), [b] Chakrabarti (unpublished observations), [c] Mondal and Chakrabarti (2002), [d] Majee et al.(1999), [e] Bhattacharyya et al. (2004), [f] Chakrabarti (1996), [g] Haque et al. (2000)

Interestingly, spectrin-bound Prodan was found to show unique fluorescence characteristics. This includes the largest blue shift in the fluorescence emission maximum (~90 nm) observed for Prodan binding to any protein (see Fig. 2). In addition, a twofold enhancement in the fluorescence intensity of Prodan in the presence of 1.5 μM dimeric spectrin was observed. Binding of Prodan to spectrin was estimated from the concentration-dependent change in the ratio of fluorescence intensity at 520 and 430 nm (corresponding to the emission maxima of Prodan in aqueous buffer and when bound to spectrin, respectively). Importantly, the stoichiometery of binding was found to be 1:1 (mol/mol). Furthermore, detailed characterization of Prodan binding to spectrin in both the dimeric and tetrameric forms of spectrin indicated that the unique Prodan binding site could localize at the self-association domain of erythroid spectrin (Bhattacharyya et al. 2004). Interestingly, the functional importance of this unique hydrophobic binding site has recently been established. Spectrin was shown to exhibit chaperone-like activity and the hydrophobic binding site at the self-association domain was implicated to be the basis of such activity (Chakrabarti and Bhattacharya 1999; Chakrabarti et al. 2001; Bhattacharyya et al. 2004; see later). The functional relevance of the binding of Prodan to spectrin is further reinforced by the observation that Prodan binding is inhibited in the presence of denaturing agents such as urea (Bhattacharyya et al. 2004).

A combined approach of docking and molecular dynamics simulation has recently been used to examine the binding mode of Prodan to spectrin. Although the X-ray or NMR structure of the self-association domain is not available, the NMR structure of the N-terminal domain of α-subunit has been reported (Pascual et al. 1997). A model of the self-association domain, based on the NMR structure of a repeat motif obtained from protein data bank (Pascual et al. 1997), has been created. Comparison of solvent

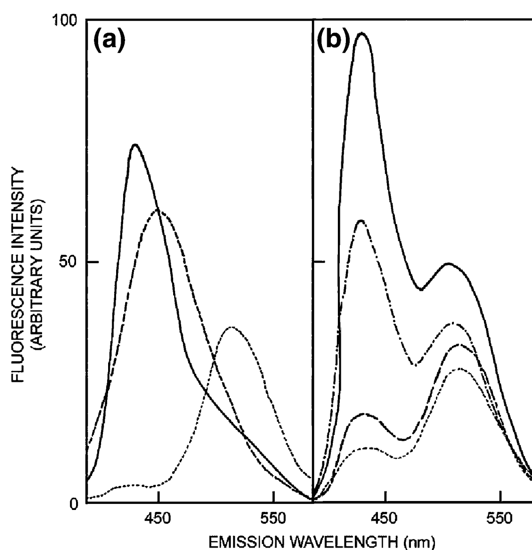

Fig. 2 Spectrin bound Prodan shows unique fluorescence characteristics. The fluorescence emission spectra of 0.5 μM Prodan in aqueous buffer (...), 45 μM bovine serum albumin (- - -), and 1.54 μM dimeric spectrin (—) are shown in (**a**). The remarkably large (~90 nm) blue shift and significant enhancement in fluorescence intensity in the presence of spectrin may be noted. The fluorescence emission spectra of 1.0 μM Prodan in the presence of increasing concentrations of spectrin are shown in (**b**). The concentrations of spectrin used were 0.13 μM (...), 0.3 μM (- - -), 1.0 μM (–·–) and 1.5 μM (—). Adapted from Chakrabarti (1996)

accessible surface area data shows that in the lowest energy binding site, Prodan is maximally shielded from water with a minimum area of ~452 Å^2 (A. Chakrabarti unpublished observations). However, the binding cavity for Prodan was found to be much greater and the percentage of hydrophobic residues in the binding subset was found to be 58%. Since Prodan is a hydrophobic ligand, such high percentage of hydrophobicity in the binding pocket leads to energetically favorable binding. Moreover, the interaction energy of the Prodan–spectrin complex displayed an energy minimum of ~–50 kcal/mol which did not appreciably improve upon further simulation (A. Chakrabarti unpublished observations).

This model is in good agreement with the fluorescence data which shows very high fluorescence intensity, the largest blue shift in emission maximum and a very large increase in the fluorescence polarization of Prodan (from 0.03 to 0.27), upon binding with spectrin (Chakrabarti 1996; Bhattacharyya et al. 2004). It was also shown from the fluorescence data that Prodan binds spectrin with a 1:1 (mol/mol) binding stoichiometry and the self-association domain holds the Prodan binding site. The widely used hydrophobic fluorescence probe ANS was found to bind with a stoichiometry of about 4 to both dimeric and tetrameric spectrin (Bhattacharyya et al. 2004). Interestingly, no specific binding site for ANS could be located from fluorescence measurements. A representative model structure of the complex between the self-association domain of erythroid spectrin and Prodan obtained by molecular dynamics simulation is shown in Fig. 3. That the high affinity binding of Prodan takes place only with dimeric (or tetrameric) spectrin is supported by the observation that Prodan binds isolated α-spectrin with significantly lower affinity ($K_d = 3.47 \times 10^{-6}$ M) (A. Chakrabarti unpublished observations).

The organization and dynamics of the Prodan binding site of spectrin has been further investigated using sensitive fluorescence approaches such as the wavelength-selective fluorescence approach. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system (Chattopadhyay 2003; Raghuraman et al. 2005). 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 red edge excitation shift (REES) (Demchenko 2002; Chattopadhyay 2003; Raghuraman et al. 2005). 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. REES arises from slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Interestingly, a large change in dipole moment (Balter et al. 1988; Samanta and Fessenden 2000), along with its hydrogen bonding capability (Samanta and Fessenden 2000), makes Prodan a suitable probe for REES effects in order to characterize the hydrophobic binding sites in spectrin. Prodan bound to spectrin was shown to display a significant REES of 9 nm (Chattopadhyay et al. 2003). Such an observation of REES for spectrin-bound Prodan suggests that when bound to spectrin, Prodan is localized in an environment where its mobility is considerably reduced. Such a result would directly imply that this region of spectrin offers considerable restriction to the reorientational motion of the solvent dipoles around the excited state fluorophore.

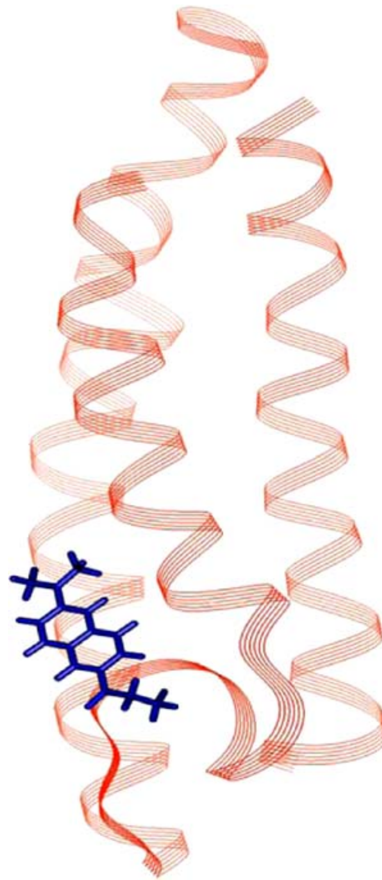

Fig. 3 A representative model structure of the complex between the self-association domain of erythroid spectrin and Prodan obtained by molecular dynamics simulation. The self-association domain was created using NMR structural data of a repeat motif obtained from the protein data bank (PDB code 1AJ3)

The hydrophobic binding sites of spectrin have been further characterized utilizing the polarity dependence of the extent of vibronic coupling of the hydrophobic fluorescent probe pyrene (Turro et al. 1986). Pyrene was shown to bind spectrin with high affinity (Table 1). The apparent dielectric constant (ϵ) of the hydrophobic pyrene binding site in erythroid spectrin was estimated using the linear relationship between the ratio of the first (373 nm) and third (394 nm) vibronic peak intensities and the dielectric constant of the bulk medium (see Fig. 4). The apparent dielectric constant of the pyrene binding site (expected to be similar to that of the Prodan binding site) on spectrin was estimated to be ~7 from an analysis of the ratios of pyrene vibronic band intensities (Haque et al. 2000).

Spectrin Tryptophans: Intrinsic Reporters of Spectrin Structure and Function

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 (Sahr et al. 1990; Winkelmann et al.

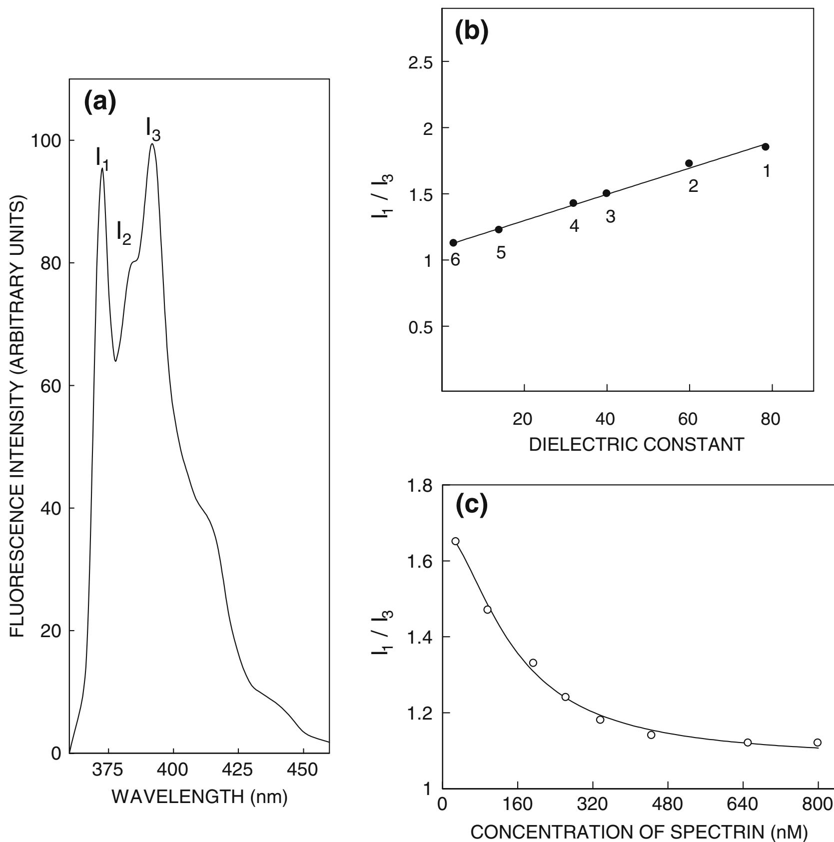

Fig. 4 Estimation of the apparent polarity of the hydrophobic binding sites in spectrin. The fluorescence emission spectrum of 2 μM pyrene in methanol is shown in (**a**). The vibronic peaks I_1 (373 nm), I_2 (384 nm) and I_3 (394 nm) are shown. The calibration curve between the fluorescence intensity ratio of I_1 and I_3, and the dielectric constant of the solvent is shown in (**b**). The points marked by numbers refer to bulk solvents of the following compositions (the numbers in parentheses denote the respective dielectric constants): (1) quartz distilled water (78.5), (2) methanol/water 40:60 v/v (60), (3) methanol/water 80:20 v/v (40), (4) methanol (32), (5) ethanol (14), and (6) diethylether (3). A plot of the ratio of (I_1/I_3) of 0.2 μM pyrene fluorescence as a function of increasing spectrin concentration is shown in (**c**). The plots in (**b**) and (**c**) are adapted and modified from Haque et al. (2000)

1990). 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 (see Fig. 1). In addition, there are five tryptophans in the actin binding domain at the amino terminus and two tryptophans at the carboxy terminus in the β subunit of the spectrin dimer. Some of these conserved tryptophans have been shown to promote folding of spectrin domains (MacDonald et al. 1994) and contribute to their thermodynamic stability (Subbarao and MacDonald 1994; Pantazatos and MacDonald 1997). 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 (Subbarao and MacDonald 1994; Kelkar et al. 2005). Many of these tryptophans are at or in the vicinity of hydrophobic patches in spectrin, which can bind hydrophobic ligands such as fatty acids and phospholipids and cause quenching of tryptophan fluorescence (Sikorski et al. 1987; Kahana et al. 1992).

As mentioned earlier, the wavelength-selective fluorescence approach offers a convenient tool to directly monitor the environment and dynamics around a fluorophore in a complex biological system. We utilized wavelength-selective fluorescence to analyze the organization and dynamics of the tryptophan residues of spectrin (Chattopadhyay et al. 2003). Figure 5 shows the shifts in the maxima of fluorescence emission of spectrin as a function of excitation wavelength. As the excitation wavelength is changed from 280 nm to 307 nm, the emission maximum of native spectrin is shifted from 338 nm to 342 nm, which corresponds to a REES of 4 nm. Such a shift in the wavelength of emission maximum with change in excitation wavelength is characteristic of the red edge effect and indicates that the tryptophans in native dimeric spectrin 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 (MacDonald et al. 1994; Subbarao and MacDonald 1994). Further, many of these tryptophans are at or in the vicinity of hydrophobic patches in spectrin which can bind hydrophobic ligands (Sikorski et al. 1987; Kahana et al. 1992; see above). As described above, the estimated apparent dielectric constant of the hydrophobic binding site is ~7 (Haque et al. 2000). The low dielectric characteristics coupled with the presence of restricted water molecules

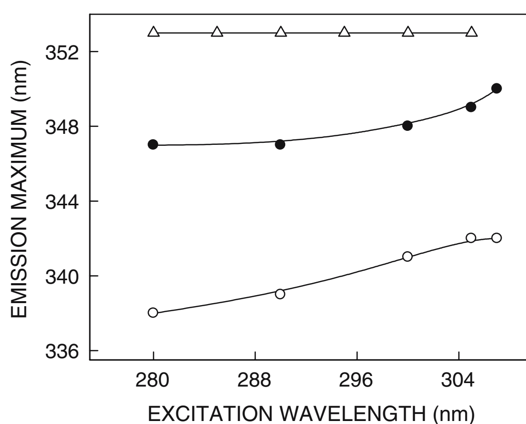

Fig. 5 Dynamics of spectrin tryptophans monitored by REES. Effect of changing excitation wavelength on the wavelength of maximum emission for native (o) and denatured (•) spectrin. The concentration of native spectrin was 0.6 μM while that of denatured spectrin was 0.4 μM. Spectrin was denatured in 8 M urea. REES obtained in case of 2 μM tubulin in 8 M urea (Δ) is shown as a control. Adapted and modified from Chattopadhyay et al. (2003) and Guha et al. (1996)

contribute in making some of these regions ideal environments for exhibiting REES and related effects (Chattopadhyay 2003).

Interestingly, spectrin displays REES even when denatured in 8 M urea (see Fig. 5). This surprising result is in contrast to earlier results where it has been shown that emission maximum for tryptophans in urea-denatured proteins such as the cytoskeletal protein tubulin do not exhibit excitation wavelength dependence (see Fig. 5; Guha et al. 1996). This is because the tryptophan residues are exposed to bulk water when denatured and therefore do not offer any restriction to the solvent (water) dipoles around them in the excited state (Demchenko 1988). The observation of REES in denatured spectrin indicates that the tryptophans are shielded from bulk solvent even when denatured and indicates local residual structure in the denatured protein. This points out the crucial structural role played by water molecules in the overall stability of spectrin. This result assumes special significance in the context of the interaction of water molecules with cytoskeletal elements which has been implicated as a major factor in maintaining the organization and function of the cytoskeleton (Leterrier 2001). The tryptophan microenvironment in spectrin is therefore characterized by unique structural and dynamic features that are maintained to a significant extent even when denatured with urea. This is further supported by analysis of fluorescence quenching data using acrylamide as a quencher (Chattopadhyay et al. 2003). Interestingly, the invariant tryptophan residue of spectrin repeats has been shown to be important for the thermodynamic stability of the repeat unit. Semi-conservative mutations of this residue to phenylalanine or tyrosine were found to considerably decrease the stability of the triple-helical bundle motif (Pantazatos and MacDonald 1997). Residual structure in an unfolded protein is thought to reside predominantly in hydrophobic clusters, where residues like tryptophan stabilize these networks through cooperative long range non-native interactions (Klein-Seetharaman et al. 2002). Although residual structure in denatured proteins has been studied before (Neri et al. 1992; Blanco et al. 1998; Bhavesh et al. 2001; Ropson et al. 2006), this observation constitutes the first demonstration of slow solvent relaxation in a completely denatured protein. The residual structural integrity that remains even after spectrin is denatured could be considered as a hallmark of a cytoskeletal protein whose main function is to provide a stable scaffold to the cell membrane. Interestingly, while urea denatured tubulin does not retain any residual structure as evidenced by the lack of REES for tubulin tryptophans (Guha et al. 1996), thermally denatured tubulin retains a significant amount of residual structure that is characterized by increased hydrophobic environments around the tryptophan residues (Mozo-Villarias et al. 1991).

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 (Lange et al. 1982; Vertessey and Steck 1989; Johnson et al. 1980). Electron microscopy studies have shown that at lower than physiological ionic strengths, spectrin appears to be elongated within the membrane skeleton (McGough and Josephs 1990). In addition, ionic strength was found to increase the cooperativity of unfolding of the spectrin dimer and isolated spectrin repeats (Lusitani et al. 1998). 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), circular dichroism (CD), and Fourier transform infrared (FTIR) studies (DeSilva et al. 1997; LaBrake et al. 1993). An analysis of the organization and dynamics of the tryptophan residues of spectrin under different conditions of ionic

strengths shows 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 (Kelkar et al. 2005). However, tryptophan accessibility to the aqueous environment, as revealed by the aqueous quencher acrylamide, displays sensitivity to the ionic strength of the medium (Subbarao and MacDonald 1994; Kelkar et al. 2005). 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 (Kelkar et al. 2005). The ionic strength induced conformational changes in spectrin therefore appear to be characterized by changes in overall accessibility of the exposed tryptophan residues while keeping the accessibility of the core tryptophans invariant.

Chaperone-like Activity of Spectrin

Spectrin shares some common interactive features with proteins that exhibit chaperone activity, and is characterized by the unique hydrophobic binding site for hydrophobic molecules through which it can interact with denatured proteins. Spectrin interacts with other proteins to establish the cytoskeletal network and self associates to form tetramers and higher order oligomers. Recently, one of us has shown that erythroid spectrin exhibits chaperone-like activity and the hydrophobic binding site at the self-association domain of spectrin has been implicated to be the basis of such activity (Bhattacharyya et al. 2004). Spectrin was shown to prevent both thermal and non-thermal aggregation of other proteins such as alcohol dehydrogenase, horseradish peroxidase and insulin (Chakrabarti and Bhattacharya 1999; Bhattacharyya et al. 2004). The dimeric spectrin and the two subunits, α- and β-spectrin prevent such aggregation even better than the well characterized chaperone Hsp90. Pairwise sequence alignment by the method of Needleman and Wunsch (1970) revealed more than 50% similarities in α-spectrin near the N-terminus with human Hsp90 and in β-spectrin near the C-terminus with human Hsp90 and *Escherichia coli* DnaJ indicating that potential chaperone-like sequences are present near the self-association domain. In both spectrin polypeptides, there are other patches of sequences, at both the termini and in the middle of the rod domain which show significant homology with well-known chaperone proteins (Bhattacharyya et al. 2004). Interestingly, *E. coli* GroEL has been shown to react with anti-β spectrin antibodies, possibly due to the presence of a single shared epitope on the surface of both proteins (Czogalla et al. 2003). One of us has earlier shown that binding of denatured horseradish peroxidase with spectrin takes place during refolding of the denatured heme enzyme leading to an inhibition of enzyme activity as shown by other known molecular chaperones (Chakrabarti et al. 2001). However, the reactivation yield was increased in the presence of Mg/ATP indicating an ATP-dependent chaperone-like activity of spectrin with a non-native heme protein as the substrate. Spectrin binds to denatured horseradish peroxidase with the dissociation constant, K_d of 16×10^{-9} M which is comparable with that of GroEL and denatured lactate dehydrogenase ($K_d = 20 \times 10^{-9}$ M). The ATP binding affinity to spectrin was also estimated to have a K_d of 1.1×10^{-3} M at 25°C (see Table 1). In this respect, spectrin showed remarkable similarity with that of the *E. coli* chaperonin, GroEL, which binds the completely denatured form of citrate synthase and lactate dehydrogenase with high affinity and the complex between GroEL and the denatured protein is destabilized upon binding of ATP (Buchner et al. 1991; Badcoe et al. 1991).

Spectrin–Membrane Interaction: Role of Specific Phospholipid Headgroups

The cellular cytoskeleton is intimately involved in cell functions such as cell–cell interactions, receptor organization, cell motility, adhesion, endocytosis, and cell division. To influence these processes the cytoskeleton must be anchored reversibly to the plasma membrane in a manner that is regulated by signaling events. Understanding the organization of the cytoskeleton in relation to the cell membrane therefore assumes relevance (Niggli 2001). There have been a number of reports from studies on cells, isolated membranes and model systems that point out direct lipid–protein interactions which could contribute to the attachment of the membrane skeleton to the membrane interior (Sikorski et al. 2000). The mechanical integrity and biconcave shape of red blood cells are maintained by a complex network of proteins on the inner surface of the cell membrane, called the membrane skeleton (Mohandas and Evans 1994; Svetina et al. 1996; Bennett 1990). The erythroid membrane skeleton is a particularly suitable model to understand the interaction of the cytoskeletal proteins with membrane phospholipids. This is due to the lack of a complex intracellular cytoskeletal network of microtubules and intermediate filaments in the mammalian erythrocyte, and the absence of intracellular organelles. The red cell skeleton is responsible for the elasticity and stability of the red cell as it negotiates the microcirculation during the passage through the circulatory system (Elgsaeter et al. 1986). Spectrin is the major constituent protein in the erythroid membrane skeleton and plays a major role with respect to its elasticity since the spectrin dimer is highly extensible (Shotton et al. 1979). The interaction of spectrin with membranes is particularly significant since it has been reported that contacts between spectrin and inner monolayer lipids are likely to contribute to the elasticity of the circulating erythrocyte (Markin and Kozlov 1988). In addition, spectrin has been reported to be involved in the maintenance of dynamic (phase-state) asymmetry in erythrocyte membranes (Williamson et al. 1982). Interestingly, spectrin has been shown to exhibit fast dynamics (diffusion) when bound to a phosphatidylcholine (PC) membrane surface, particularly when the membrane is in the fluid phase (O'Toole et al. 1999). This was thought to be a result of multiple weak spectrin–lipid interactions along the length of the spectrin molecule that are constantly broken and reformed (Mc Kiernan et al. 1997; O'Toole et al. 1999).

Studies on isolated membranes and model membrane systems indicate direct contribution of lipid–protein interactions in the attachment of the spectrin-based membrane skeleton to the bilayer membrane (Sikorski et al. 2000). Early spectrinphospholipid binding studies suggested that spectrin binds to zwitterionic phospholipids such as PC and phosphatidylethanolamine (PE) with greater affinity than to negatively charged phospholipids such as phosphatidylserine (PS) (Mombers et al. 1980; Sikorski et al. 2000). Interestingly, while it is known that membrane–spectrin interactions are dependent on membrane order (Mombers et al. 1977), it has been very recently reported that the way membrane order is regulated is also important. Therefore, changes in membrane order either due to increased acyl chain length or due to increased cholesterol content are found to have specific effects on spectrin binding (Diakowski et al. 2006). The equilibrium dissociation constants obtained from different laboratories (Bitbol et al. 1989; Bialkowska et al. 1994; O'Toole et al. 2000) show a wide range of values ranging from 89 nM to 2,700 nM, depending on the ionic strength and pH of the medium, the type of phospholipids used, and the specific technique used (Sikorski et al. 2000). Interestingly, the erythrocyte membrane contains significant amounts of PE and

PS (~20% PE and 10% PS by weight) which are almost exclusively localized in the inner leaflet (Op den Kamp 1979; Florin-Christensen et al. 2001; Koumanov et al. 2005).

Fluorescence quenching of spectrin tryptophans represents a convenient approach to monitor the interaction of phospholipids with spectrin. Such quenching interactions are usually short-ranged (~5–7 Å) and require molecular contact (Eftink 1991). The fluorescence lifetime of tryptophan residues in proteins is typically in the nanosecond range (Beechem and Brand 1985). Thus, the time scale of the interaction between tryptophan residues and the quencher would be faster than that for the exchange of phospholipids at the boundary (annulus) of the protein (London and Feigenson 1981). The phospholipid-induced quenching of spectrin tryptophan fluorescence is therefore a static effect, i.e., the distance between the fluorophore and quencher does not change during the lifetime of the excited state of tryptophan (Chattopadhyay 1992). This type of quenching is often characterized by the invariance of fluorescence lifetime in the presence of the quencher (Birks 1970). For example, the fluorescence lifetime of spectrin tryptophans has been shown not to be significantly affected in the presence of phospholipids (Ray and Chakrabarti 2004). Relative fluorescence quenching obtained from experiments involving different phospholipids can be used to measure relative binding constants of phospholipids.

Although a number of studies have been carried out on the interaction of spectrin with phospholipids from natural membranes, liposomes and monolayer lipid films under a variety of conditions (Haest 1981; Subbarao et al. 1991; MacDonald 1993; Sikorski et al. 2000), very few studies have examined the surface density dependent interactions of phoshpolipids with spectrin. Recently, surface density dependent high affinity binding of PE to spectrin has been reported utilizing quenching of spectrin tryptophan fluorescence by neighboring phospholipids using gel and fluid phase model membranes (Ray and Chakrabarti 2004). This work is based on an earlier observation that the addition of phospholipid suspensions to spectrin causes quenching of its intrinsic tryptophan fluorescence (Sikorski et al. 1987; Diakowski et al. 1999). The surface density-dependent spectrin binding to PC/PE vesicles was directly evaluated from the increase in the extent of quenching of spectrin tryptophan fluorescence with increasing concentrations of the phospholipid vesicles (Ray and Chakrabarti 2004). The apparent molecular masses of the membrane vesicles prepared from pure synthetic phospholipids were determined from the diameter of the vesicles and other geometric parameters of the phospholipids from the existing literature. Interestingly, the dissociation constants of spectrin binding to phospholipid vesicles were found to increase with increasing surface density of PE in PC/PE vesicles. However, a stronger association between spectrin and PE was observed in pure PE membranes. Fluorescence quenching and electron microscopic measurements (see Fig. 6) showed that the PE-binding site of spectrin is localized at one end of dimeric spectrin, either at the self-association domain/or at the actin binding domain. Figure 6 shows a proposed model depicting the orientation of spectrin in membranes containing PC and PE (Ray and Chakrabarti 2004).

Conclusion and Future Perspectives

Spectrin research appears to have reached an interesting crossroad. The classical view of spectrin has been that of a structural protein. However, this view has undergone a paradigm shift with the realization of possible diverse biological roles of spectrin besides

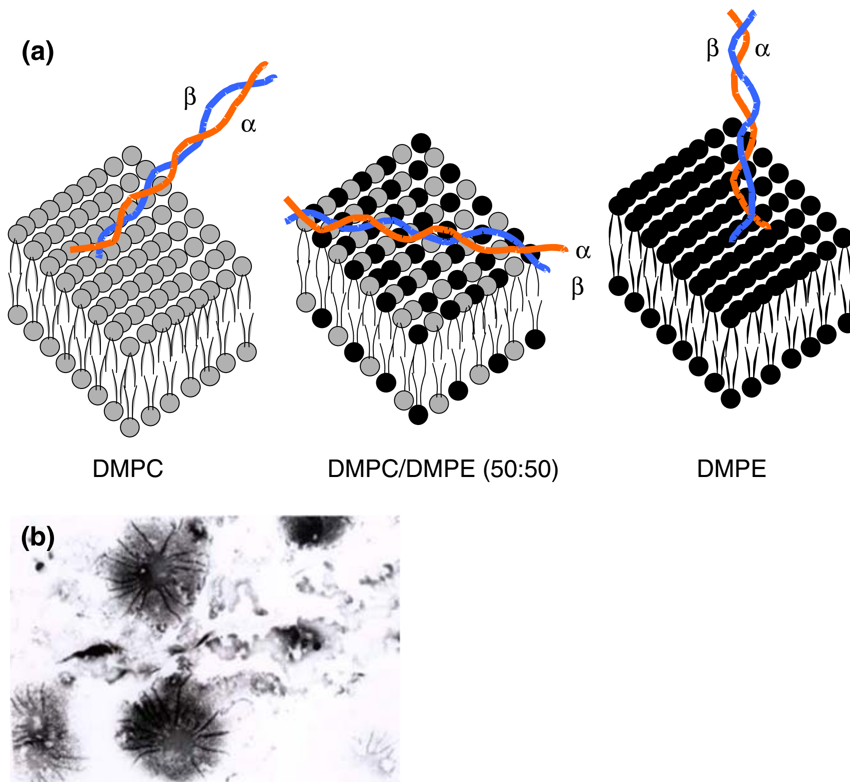

Fig. 6 Orientation of the worm-like spectrin dimer in bilayers of various headgroup lipids. (**a**) Schematic representation of the orientation of spectrin in PC (grey head groups), PC/PE (1:1) and PE (black head groups) membranes. Note that the PEbinding sites localize at the terminal end of the spectrin dimer and PE can therefore accommodate a large number of spectrin dimers. (**b**) Electron micrograph of spectrin dimer (0.1 μM) attached to PE membranes. Adapted and modified from Ray and Chakrabarti (2004)

its primary role in the erythrocyte membrane skeleton. These attributes include chaperon-like activity (Bhattacharyya et al. 2004), protein trafficking (Beck and Nelson 1996; De Matties and Morrow 2000), endocytosis (Phillips and Thomas 2006) and structural integrity resistant to denaturation (as monitored by REES, Chattopadhyay et al. 2003). Such structural integrity that is resistant to denaturation could be characteristic of cytoskeletal proteins whose main function is to provide a stable scaffold to the cell membrane. On the other hand, the role of the interaction of spectrin with various proteins in certain types of disease processes has started to emerge as an important factor in pathogenic conditions. For example, the affinity of binding of spectrin to hemoglobin and its variants has been shown to increase in β-thalassemic patients (Datta et al. 2003). In summary, the implications of spectrin mediated interactions in cellular physiology in healthy and diseased states are beginning to be unraveled, and future research in this area appears promising.

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