Binding of polarity-sensitive hydrophobic ligands to erythroid and nonerythroid spectrin: fluorescence and molecular modeling studies

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We have used three polarity-sensitive fluorescence probes, 6-propionyl 2-(N,N-dimethyl-amino) naphthalene (Prodan), pyrene and 8-anilino 1-naphthalene sulfonic acid, to study their binding with erythroid and nonerythroid spectrin, using fluorescence spectroscopy. We have found that both bind to prodan and pyrene with high affinities with apparent dissociation constants (\(K_d\)) of .50 and .17 \(\mu\)M, for prodan, and .04 and .02 \(\mu\)M, for pyrene, respectively. The most striking aspect of these bindings have been that the binding stoichiometry have been equal to 1 in erythroid spectrin, both in dimeric and tetrameric form, and in tetrameric nonerythroid spectrin. From an estimate of apparent dielectric constants, the polarity of the binding site in both erythroid and nonerythroid forms have been found to be extremely hydrophobic.

Thermodynamic parameters associated with such binding revealed that the binding is favored by positive change in entropy. Molecular docking studies alone indicate that both prodan and pyrene bind to the four major structural domains, following the order in the strength of binding to the Ankyrin binding domain > SH3 domain > Self-association domain > N-terminal domain of \(\alpha\)-spectrin of both forms of spectrin. The binding experiments, particularly with the tetrameric nonerythroid spectrin, however, indicate more toward the self association domain in offering the unique binding site, since the binding stoichiometry have been 1 in all forms of dimeric and tetrameric spectrin, so far studied by us. Further studies are needed to characterize the hydrophobic binding sites in both forms of spectrin.

**Keywords:** hydrophobic ligand; erythroid spectrin; nonerythroid spectrin; fluorescence; molecular modeling

**Introduction**

Red blood cell integrity and flexibility is controlled through mutual interactions of proteins, forming a meshwork known as membrane skeleton (Bennett, 1992). The principal constituent of the erythroid cytoskeleton is spectrin that forms a filamentous network on the cytoplasmic surface through interaction with other membrane skeletal proteins, such as actin, ankyrin, adducings, and band 4.1 (Hartwig, 1994, 1995). Spectrin performs a major role in maintaining the elastic deformability of red blood cell, during circulation. Several blood diseases, such as hereditary spherocytosis, are related to the deformability and abnormal shape of erythrocytes caused by mutations in erythroid spectrin (Delaunay & Dhermy, 1993; Gallagher et al., 1997; Wichterle, Hanspal, Palek, & Jarolim, 1996). Spectrin is also involved in the maintenance of Golgi structure and function (DeMatteis & Morrow, 2000). Erythroid spectrin has also been reported to exhibit chaperon like-activity and participate in sorting of proteins (Beck & Nelson, 1996; Bhattacharyya, Ray, Bhattacharyya, & Chakrabarti, 2004).

Spectrin is a large heterodimer protein, composed of \(\alpha\)- and \(\beta\)-subunit, with large molecular weights of 280 and 246 kDa, respectively. The two subunits, \(\alpha\)- and \(\beta\)-spectrin, are associated laterally through the carboxy terminal of \(\alpha\)-spectrin and amino terminal of \(\beta\)-spectrin to give a 100-nm-long rod-shaped, worm-like heterodimeric molecule (Spechier et al., 1993; Ursitti, Kotula, Desilva, Curtis, & Speicher, 1996). The heterodimer, in turn, forms tetramer or higher oligomer with the N-terminal region of \(\alpha\)-spectrin and C-terminal of \(\beta\)-spectrin, generating the self association domain, connected through \(\alpha\)-helical linker (Begg, Morris, & Ralston, 1997). The additional structural motifs of spectrin are the actin-binding domain, SH3 domain, pelestrin homology domain, and a calmodulin-like domain, allowing...
spectrin to take part in a number of physiological events through protein–protein interaction and protein–membrane interactions.

The other form of spectrin, found in nonerythroid tissues, bears a high sequence homology with the erythroid spectrin. Nonerythroid spectrin also, in a similar manner, forms heterodimer in an antiparallel side-to-side orientation, forming a rod-shaped molecule. Although the N-terminal region of α-subunit and C-terminal region of β-subunit of nonerythroid spectrin exhibit about 60 and 70% similarity, respectively, with erythroid spectrin (Li & Fung, 2009; Mehabboob et al., 2003), the two spectrin isoforms are quite different in terms of their structure and function. Crystallographic structural data reveals that the tetramerization site or the self-associating domain of nonerythroid α-spectrin is different from that of erythroid spectrin (Mehaboob et al., 2010). This is supported by the fact that the heterodimer of nonerythroid spectrin forms tetramer about 15 times stronger than erythroid spectrin (Begg et al., 1997). Nonerythroid spectrin is more rigid and thermally stable than the erythroid spectrin (An et al., 2006), and interacts more strongly with anionic lipid membrane than erythroid spectrin (Diakowski & Sikorski, 1995, 2002).

Extrinsic fluorescent dyes are extensively used in studies to characterize the folding intermediates, probing surface hydrophobicity, polarity of microenvironment, and aggregation of proteins. The most extensively utilized fluorescence probe for such studies is anionic sulphonic acid class, such as 8-anilonaphthalene-1-naphthalenesulfonic acid (ANS), bis-ANS, and 6-(p-toluidinyl)naphthalene-2-sulfonate (Harskard & Li-chan, 1998). The emission spectra of ANS are very sensitive to environmental polarity. In polar environment, the emission intensity, quantum yield, and fluorescence lifetime are very low, which substantially increase the association with large blue shift in hydrophobic solvent or when bound to the hydrophobic surface of the protein (Dainel & Waber, 1966; Krik & Klimchuk, 2007). Although the increase of fluorescence intensity and lifetime of ANS is attributed to hydrophobic interaction, the crystal structure of protein–ANS complex show contributions of electrostatic as well as hydrophobic interactions for ANS binding (Gasymov & Glasgow, 2007; Matulis & Lovrien, 1998; Schonbrunn, Eschenburg, Lugers, Kabsch, & Amerhein, 2000).

In the present work, we have used neutral ligands as more appropriate probes for the determination of protein hydrophobicity. Prodan (6-propinyl-2-(N,N-dimethylamino) naphthalene), a naphthalene-based fluorescence probe, introduced by Weber and Farris, has been used to determine the polarity of biological environment (Weber & Farris, 1979). Due to the presence of both electron donating and withdrawing group in the naphthalene ring, prodan exhibits a large change of dipole moment by intramolecular charge transfer in the excited state, leading to the largest blue shift depending on the polarity of the microenvironment in the emission maxima. Prodan has been also used as a probe in lipid membranes and as marker to estimate the polarity of heme-binding pocket in apomyoglobin (Bondar & Rowe, 1999; Macgregor & Weber, 1986; Mazumdar, Parrack, & Bhattacharyya, 1992). Pyrene is another suitable neutral probe to investigate the structure and conformation of lipids bilayers, proteins, and nucleic acids, often used in membrane biophysics and cell biology (Bains, Patel, & Narayanaswami, 2011; Tamamizukato et al., 2006). The emission spectrum of monomeric pyrene is very sensitive to the polarity of microenvironment. The π-π* transitions reflected in the emission spectra of pyrene exhibit five well-resolved vibronic bands between 370 and 400 nm. During increase in polarity of solvent, the vibronic state and electronic state of pyrene are coupled; the first vibronic band (I1) undergoes significant enhancement in emission intensity compared to the third band (I3). By measuring the ratio of the emission intensities, I1/I3, in solvents of different polarity, Py-scale solvent polarity could be estimated in the vicinity of the probe (Dong & Winnick, 1982; Winnick, 1993).

Spectrin has large number of hydrophobic stretches in its polypeptide sequence, which could bind hydrophobic ligand, fatty acid, and phospholipids (Isenberg, Kenna, Green, & Gratzer, 1981; Kahana, Pinder, Smith, & Gratzer, 1992; Ray & Chakrabarti, 2004). The hydrophobic interactions are crucial contributors to the conformational stability and functionality of spectrin, and are important for protein–protein, protein–membrane interaction. Previous studies from our group showed that erythroid spectrin could bind prodan, pyrene, and ANS with binding stoichiometry 1, 1, and 5, respectively (Bhattacharyya et al., 2004; Bhattacharyya, Mukhopadhay, & Chakrabarti, 2006; Chakrabarti, 1996). We have used both erythroid spectrin and the nonerythroid spectrin or fodrin, which are structurally homologous but functionally different, and have carried out a comparative study of hydrophobic ligand binding of both the spectrin by fluorescence spectroscopy and molecular docking approach. Our fluorescence study indicates that both the protein bind hydrophobic ligand with similar affinity and binding stoichiometry. Molecular modeling study alone revealed that the unique binding site of prodan and pyrene could be localized, in either the SH3 domain or the ankyrin binding domain of both erythroid and nonerythroid spectrin. Previous crystallographic study (Stabach et al., 2009) and the present modeling work support that ankyrin-binding domain is more suitable to accommodate these hydrophobic ligands. However, the binding experiments also reveal the binding stoichiometry to be 1, particularly for the tetrameric nonerythroid spectrin, and indicate toward the self-associating domain to offer...
the unique binding site. Thermodynamic parameters indicate that binding of prodan and pyrene are entropically favorable in both forms of spectrin. However, significant differences were reflected in the thermodynamic parameters with dominant entropic contribution in the binding of prodan and pyrene to the erythroid/nonerythroid spectrin, clearly showing enthalpy–entropy compensation in the binding process.

Materials and methods

Materials

Tris, KCl, phenyl methylsulfonyl fluoride (PMSF), dithiothreitol (DTT), EDTA, imidazole, MgCl₂, and NaCl were obtained from Sigma chemical company. Pyrene was obtained from Aldrich and was further purified over silica-gel column, using cyclohexane as solvent. Prodan & ANS were obtained from molecular probe. Deionized water from Milli Q (Millipore Corporation, USA) was used for the preparation of buffer and all other solutions.

Isolation and purification of erythroid spectrin

Clean, white ghosts from goat blood were prepared by hypotonic lysis in 5 mm phosphate and 1 mm EDTA containing 20 µg/ml of PMSF at pH 8.0 (lysis buffer) and spectrin dimer was purified from ovine erythrocyte, as described earlier (Majee, Dasgupta, & Chakrabarti, 1999; Ray & Chakrabarti, 2003). After washing the membrane thoroughly in lysis buffer, the band-6 depleted ghosts were resuspended in spectrin removal buffer (.2 mM sodium phosphate buffer, .1 mM EDTA, .2 mM DTT, and .2 mM PMSF at pH 8.0) and incubated 37 °C for 30 min. The crude spectrin was collected in the supernatant after centrifugation. Spectrin was then purified after concentrating with 50% ammonium sulphate precipitation followed by Sepharose CL-4B column chromatography and stored in buffer, containing 1 mM sodium phosphate, 20 mM KCl containing .2 mM DTT at pH 8.0, and .2 mM PMSF. The purity of the preparation was checked by 8% SDS-Polyacrylamide gel electrophoresis, under reducing condition (shown as Figure S1 supplementary material). Before all the fluorescence experiments, nonerythroid spectrin was dialyzed against the buffer, containing 10 mM Tris and 20 mM KCl pH 7.8. Nonerythroid spectrin concentration was determined by Bradford method with bovine serum albumin as a standard (Bradford, 1976).

Binding of Prodan to spectrin by fluorescence measurements

Steady-state fluorescence experiment of prodan and ANS were performed as described previously (Bhattacharyya et al., 2004; Ray, Bhattacharya, & Chakrabarti, 2005) using a Fluoromax-3 spectrophotometer, using a 10-mm path-length cuvette. Prodan binding was done using .2 µM prodan, to which increasing amount of fodrin were added. Fluorescence spectra of prodan were recorded upon excitation at 360 nm with band-pass slit of 5 nm each for excitation and emission channels. The buffer used in the present study contained 10 mM Tris-HCl and 20 mM KCl at pH 7.8. The sample temperature was maintained at 25 °C for all the experiments, unless mentioned otherwise. The concentrations of prodan and ANS in dimethylformamide were determined using molar absorbance of 18,000 at 360 nm and 7800 at 372 nm, respectively (Bhattacharyya et al., 2004).

The apparent dissociation constants were determined by three different methods. First, a model-independent method was used, and the apparent dissociation constants (Kₐ) were determined by non-linear curve fitting, based on the equilibrium L + P → L – P, where L and P are the ligands, ANS and prodan and the protein, respectively. The data points obtained from fluorometric
titration were analyzed by fitting the two equations given below (Ali et al., 1999; Bhattacharyya et al., 2004). All the experimental points for the binding isotherm were fitted by least squares analysis, using Microcal Origin software package (version 8.0, Microcal software Inc., Northampton, MA). The binding stoichiometries of the complex of nonerythroid spectrin, with prodan and ANS, were estimated from the intercept of two straight lines of the plot of $\Delta F/\Delta F_{\text{max}}$ against the mole fraction nonerythroid spectrin and ANS, after non-linear fitting as follows:

$$K_d = [(C_0 - \Delta F/\Delta F_{\text{max}})C_0][(C_p - \Delta F/\Delta F_{\text{max}})C_0]/(\Delta F/\Delta F_{\text{max}})C_0 \quad (1)$$

$$C_0(\Delta F/\Delta F_{\text{max}})^2 - (C_0 - C_p + K_d)(\Delta F/\Delta F_{\text{max}}) + C_p = 0 \quad (2)$$

In both the Equations (1) and (2), $\Delta F$ is change in fluorescence emission intensity or the ratio $I_{330}/I_{320}$ ($\lambda_{\text{ex}} = 360$ nm) for each point of the titration curve, and $\Delta F_{\text{max}}$ is the change in fluorescence intensity, when the protein is completely bound to prodan. $C_p$ is the concentration of protein and $C_0$ is the initial concentration of prodan. The double reciprocal plot was used to determine $\Delta F_{\text{max}}$, and also the apparent binding constant ($K_{\text{app}}$), using equation shown below:

$$1/\Delta F = 1/\Delta F_{\text{max}} + 1/[K_{\text{app}}\Delta F_{\text{max}}(C_p - C_0)] \quad (3)$$

The linear double reciprocal plot of $1/\Delta F$ against $1/(C_pC_0)$ is extrapolated to the ordinate, to obtain the value of $\Delta F_{\text{max}}$. The apparent binding constant is the inverse of apparent dissociation constant ($K_0$). This approach is based on the assumption that the change of the ratio of emission intensities of prodan is proportional to the concentration of nonerythroid spectrin.

The Scatchard equation (Scatchard, Coleman, & Shen, 1957) was also used to estimate the intrinsic binding constant ($K_0$) and the binding stoichiometry ($n$):

$$r/C_t = K_0(n - r) \quad (4)$$

where $r = C_b/C_p$, when $C_b$ is the concentration of the spectrin-bound prodan and $C_p$ is the input concentration of erythroid and nonerythroid spectrin, $n$ is the binding stoichiometry, expressed as the number of prodan bound per erythroid and non-erythroid spectrin, and $K_0$ is the intrinsic binding constant ($K_{\text{app}} = K_0 \times n$). The concentration of the bound prodan ($C_t$) was determined by normalizing the input concentration of prodan with $\Delta F/\Delta F_{\text{max}}$ (Ray et al., 2005). The Scatchard plot was obtained by plotting $r/C_t$ against $r$, where $C_t$ is $(C_0 - C_b)$, and the best-fit straight line through the experimental data points led to the determination of binding constant, $K_0$, and the stoichiometry, $n$.

### Pyrene binding to erythroid and nonerythroid spectrin

Pyrene binding to erythroid and nonerythroid spectrin was done using $0.2 \mu M$ pyrene, to which increasing concentrations of erythroid and non-erythroid spectrin were added. Pyrene concentration in methanol was determined using molar absorbance coefficient ($\epsilon$) 54,000 M$^{-1}$ cm$^{-1}$ at 337 nm. Excitation wavelength used was 335 nm and pyrene emission was monitored between 350 and 450 nm in buffer, containing 10 mM Tris and 20 mM KCl at pH 7.8. Pyrene binding to erythroid and non-erythroid spectrin was estimated, following the above methods, following the change in the ratio of the emission intensities of the two vibronic bands, $I_1/I_3$, against increasing concentrations of spectrin (Haque, Roy, & Chakrabarti, 2000).

### Fluorescence polarization and lifetime measurements

Steady-state fluorescence polarization measurements were performed using a Cary Eclipsed polarization accessory. Polarization values were calculated from the equation (Chen & Bowman, 1965) shown below:

$$P = I_{VV} - GI_{VH} \rightarrow I_{VH} + GI_{VH} \quad (5)$$

where $I_{VV}$ and $I_{VH}$ are the measured fluorescence intensities, with excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented. $G$ is the grating correction factor and is equal to $I_{VV}/I_{HH}$. For polarization measurement of free prodan and prodan bound spectrins, excitation was made at 360 nm with emission wavelengths at 520 and 430 nm, respectively. Similarly, for ANS and ANS bound spectrin, emission wavelengths have been 530 and 470 nm, respectively, upon excitation at 372 nm.

Fluorescence lifetime measurements were made by a time-correlated single-photon counting Flurornax-3 spectrophotometer, using NanoLED as a light source. To optimize the signal to noise ratio, 10,000 counts were collected in the peak channel. The excitation wavelength used, were 295 nm for tryptophan, 360 nm for prodan, and 370 nm for ANS, respectively. Emission was monitored at 340 nm for prodan-bound erythroid and nonerythroid spectrin and 520 for free prodan. Emission wavelength was set at 340 nm for tryptophan and 470 for ANS-bound erythroid and nonerythroid spectrin. All experiments were performed using excitation and emission silt 2 and 3, respectively. The observed fluorescence transient were fitted by using a non-linear least squares fitting procedure to a function $X(t) = \int_0^t (E(t')R(t-t'))dt'$, comprising convolution of the IRE (E(t)) with a some of exponentials ($R(t) = A + \sum_{i=1}^N B_i e^{t/i}$) with pre-exponential factors ($B_i$), characteristic lifetime ($r$), and
background ($A$). Relative contributions from each component were obtained using tri- or bi-exponential fitting, and were finally expressed by the following equation:

$$a_n = B_n/\Sigma_i = 1^N B_i$$  \hspace{1cm} (6)

The mean lifetime for the decay curves were calculated from the decay times and relative contribution of the each component, using the following equation: (Lakowicz, 2006).

$$\tau_0 = \Sigma \tau x_i^2/\Sigma \tau x_i$$ \hspace{1cm} (7)

where ($\tau_0$) is the mean lifetime.

### Molecular modeling of the docking of prodan & pyrene to the structural domains of spectrin

We have used molecular docking approach to elucidate binding mode of prodan and pyrene with different structural domains of erythroid and nonerythroid spectrin. As the crystal structure or NMR structure of all the domains of erythroid and nonerythroid spectrin are not available, we built the homology model of different domains of both spectrins, by using Modweb (http://modbase.combio.uccsf.edu/ModWEB20html/Modweb.html). The amino acid sequences of different domains of erythroid and nonerythroid spectrin are taken from SWISS PROT.

The self-association domain of nonerythroid spectrin and SH3 domain of erythroid spectrin were created using the structure of self-association domain erythroid (3LBX.pdb) and nonerythroid spectrin SH3 domain (1NEG.pdb) as the template structure. The other domains of the spectrin, such as self association domain of spectrin (3LBX.pdb) and 3-N terminal region of erythroid (1OWA.pdb) of nonerythroid spectrin (3F31.pdb) and SH3 domain of nonerythroid spectrin (1NEG.pdb) and ankyrin-binding domain of erythroid (3F7A.pdb) of nonerythroid spectrin (3edvA.pdb), were studied using their respective crystal or NMR structure (summarized in supporting information Table S2). Energy minimization of the models was performed using GROMACS OPLS force field (Jorgensen & Rives, 1988). The three-dimensional structure of the ligand was built with the help of Biopolymer module of Insight-II (version 98 Accelrys Inc.,USA), running on a Silicon Graphics O2 workstation. Docking studies were performed with Auto Dock 4.2 (Morris et al., 2009) software packages to calculate the interaction between ligand and different domains of both spectrins. Lamarckian genetic algorithm, implemented in autodock, was applied to calculate the possible conformation of the hydrophobic ligand that binds to different domains of protein. The size of the grid box was 70, 90, and 60 along $X$, $Y$, and $Z$-axes, with 1.00 Å grid space and total number of grid points as 60,5625. During the docking process, a maximum of 10 conformers were considered for the ligand to bind protein, and the lowest binding free energy conformer was used for further analysis. The lowest energy binding domain was visualized using PyMOL Molecular Graphics System (Delano, 2002).

### Evaluation of thermodynamic parameters

The thermodynamic parameters associated with the binding of prodan and pyrene to erythroid and nonerythroid spectrin was calculated using the following equation:

$$\ln K_{app} = -(\Delta H/RT) + \Delta S/R$$  \hspace{1cm} (8)

$$\Delta G = \Delta H - T \Delta S$$  \hspace{1cm} (9)

where $R$ and $T$ are the universal gas constant and absolute temperature. The apparent binding constant ($K_{app} = 1/K_d$) was measured at different temperature to evaluate $\Delta H$ and $\Delta S$ from the slope and intercept in a plot of $\ln K_{app}$ against $1/T$.

### Results and discussion

#### Effect of erythroid and nonerythroid spectrin on the emission spectra of Prodan

Fluorescence of prodan has been widely used to estimate the polarity of the biological environment. Upon excitation at 360 nm, prodan shows bright a emission spectra in the range of 460–600 nm, with maximum emission at 520 nm in aqueous buffer (Weber & Farris, 1979). We have previously shown that the emission spectra of prodan are blue shifted to 430 nm along, with an enhancement of fluorescence intensity at 430 nm upon binding to spectrin (Bhattacharyya et al., 2004; Chakrabarti, 1996). Nonerythroid spectrin also binds prodan with similar fluorescence characteristics associated with binding, as shown in Figure 1. Figure 2 shows the representative binding isotherms of binding of prodan and pyrene to erythroid and nonerythroid spectrin at 25 °C, as plots of normalized changes in the ratio of fluorescence intensities (for prodan & pyrene) or the enhanced intensities against the concentration of erythroid or nonerythroid spectrin. The addition of both spectrins cause hypsochromic shift of prodan emission maxima, indicating that the microenvironment around the probe is quiet different from that in aqueous buffer (Balter, Nowak, Pawelkiewicz, & Kowaleczyk, 1988). This is also found when prodan partitions in phospholipid bilayer during transition of phospholipids from crystalline to gel phase (Bondar & Rowe, 1999). Such a large blue shift indicates solubilizing of prodan in the hydrophobic cavity of the protein. Encapsulation of prodan in the hydrophobic pocket of the protein causes destabilization of the
excited-state dipole moment of the probe, in the proteinous medium compared to aqueous buffer, resulting in enhancement of the quantum yield of prodan. Binding constants were determined by following three independent methods of non-linear curve fitting, double reciprocal plot (Figure 2), and Scatchard plots, as shown in the supporting information, Figure S2. The binding isotherms clearly indicate that nonerythroid spectrin forms 1:1 complex with prodan and pyrene, with the exception of ANS (not shown) with apparent dissociation constants ($K_d$) similar to erythroid spectrin, summarized in Table 1. Prodan, though not widely used for probing proteins due to its poor solubility and lower affinity (Mazumdar et al., 1992), shows great promise as the suitable probe for conformation studies of proteins of spectrin family (Bhattacharyya et al., 2004).

**Pyrene binding to erythroid and non-erythroid spectrin**

The shape of the emission spectra of monomeric pyrene is very sensitive to environment polarity. In nonpolar solvents, pyrene exhibits well-resolved vibronic bands at 373 nm ($I_1$), 379 nm ($I_2$), 384 nm ($I_3$), 389 nm ($I_4$), and 394 nm ($I_5$); but, in polar or aqueous medium, mostly three bands at 373 nm ($I_1$), 384 nm ($I_3$), and 394 nm ($I_5$) are observed. We have determined the polarity of the pyrene-binding site of erythroid and nonerythroid
spectrin using Py-scale, as reported earlier by our and other groups (Haque et al., 2000; Tamamizukato et al., 2006; Tedeschi, Mohwald, & Kirsten, 2001). In aqueous buffer, the vibronic bands at 373 and 394 nm are well resolved, but the band 384 nm (Py value = 1.87) is barely observed. In methanol, the intensity of the vibronic band at 384 nm (I_3) increases (Py value = 1.35), but in nonpolar solvents like cyclohexane, intensity of the vibronic band I_3 is greatly enhanced (Py value = .58) (Dong & Winnick, 1982). Thus, a small change in Py value can reflect large differences in microenvironment polarity of probe. A calibration curve is created between I_1/I_3 ratio against apparent dielectric constant of various solvents (data not shown) to gain information on apparent polarity of pyrene in various proteinaceous environments. The fluorescence emission spectra of monomeric pyrene at three different concentrations of the two proteins were recorded upon excitation at 335 nm (shown in Supporting Information Figure S1). Only at higher concentrations of the proteins, the vibronic bands are well resolved, showing fine vibronic structures of pyrene emission. The plot of the ratio of intensities (I_1/I_3) vs. concentration of the protein is used to estimate binding constant for molecular complexes of pyrene with erythroid/nonerythroid spectrin (Supporting Information Figure S2). Binding data reveals that both forms of spectrin bind pyrene with comparable affinity, with binding stoichiometry equal to 1 pyrene per tetrameric erythroid/nonerythroid spectrin. The apparent dielectric constant experienced by pyrene in erythroid and nonerythroid spectrin is estimated to be in between 7 and 8.

**Estimation of thermodynamic parameters associated with the binding of hydrophobic ligands to erythroid and nonerythroid spectrin**

In order to identify molecular forces involved in the binding of prodan and pyrene with spectrin, the thermodynamic parameters associated with such binding were determined in both erythroid and nonerythroid spectrin by studying the binding at different temperatures. The estimated binding constants were plotted following Van’t Hoff’s equation Equation (7), as shown in Figure 3. During the binding process, protein–ligand complex experiences different types of forces, e.g. hydrophobic force, hydrogen bonding, van der Waal’s force, and electrostatic forces (Ross & Subramanian, 1981). The thermodynamic parameters determined from Van’t Hoff’s equation indicate the negative changes in free energy (∆G) of formation of the prodan/pyrene–erythroid/nonerythroid spectrin complexes as spontaneous. The free energy of binding of such neutral hydrophobic ligand with erythroid and nonerythroid spectrin does not vary with temperature, but there is a striking difference in the magnitude of change in enthalpy and entropy, clearly indicating enthalpy–entropy compensation mechanism associated with solvent recognition accompanying the protein ligand interaction (Cleary & Glick, 2003; Das et al., 2009). Thermodynamic parameters, summarized in Table 2, show that both erythroid and nonerythroid spectrin bind prodan with positive changes in enthalpy (∆H) and entropy (∆S), indicating that the interactions between prodan and erythroid/nonerythroid spectrin are endothermic and entropy driven in nature. Positive contributions of ∆S arise mainly from hydrophobic effect of removal of water molecules from the binding surface, as well as conformational restriction due to complex formation (Torigoe et al., 1995). Although the positive ∆H value is unfavorable, the large ∆S terms may provide the major driving force for the association of prodan with erythroid and nonerythroid spectrin. Sturtevant estimated that the total entropic contribution of a ligand binding process comes from hydrophobic and vibrational contributions, which are positive and negative, respectively (Sturtevant, 1977). A further study of ∆H and ∆S values between erythroid and nonerythroid spectrin clearly points out that binding of prodan to the nonerythroid spectrin is associated with larger (positive) changes in both enthalpy and entropy, compared to erythroid spectrin. Interestingly, the binding of pyrene to both forms of spectrin is associated with negative changes in ∆H and positive changes in ∆S, which suggest that the binding process is exothermic and entropically favorable (Table 2). In case of pyrene binding, high enthalpy changes balance out the effect of smaller ∆S associated with pyrene binding to nonerythroid spectrin. The negative enthalpy value of pyrene and erythroid, nonerythroid spectrin complex

| Table 1. Apparent dissociation constants (K_d in μM) of prodan and pyrene to erythroid and nonerythroid spectrin at 25 °C, estimated by three different methods. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Double reciprocal plot | Non-linear curve fitting prodan pyrene | Scatchard plot |
|                  | Prodan | Pyrene | Prodan | Pyrene | Prodan | Pyrene |
| Erythroid spectrin | 0.50 ± 0.1 | 0.04 ± 0.02 | 0.43 ± 0.2 | 0.07 ± 0.04 | 0.38 ± 0.13 | 0.18 ± 0.4 |
| Non-erythroid spectrin | 0.171 ± 0.02 | 0.02 ± 0.01 | 0.16 ± 0.02 | 0.05 ± 0.02 | 0.14 ± 0.1 | 0.15 ± 0.3 |
indicates that non-covalent interactions play a major role in the complex formation. Although the binding energy of both prodan and pyrene are comparable, difference in the ΔH and ΔS indicate enthalpy–entropy compensation mechanism (Komath, Kenoth, & Swamy, 2001). Although the the apparent dissociation constants are comparable, the binding mechanisms could be different. Our objective of studying the binding of prodan and pyrene to both forms of spectrin has been to probe conformational differences between the two very similar proteins, localized in two entirely different cell types. We aim to correlate any differential conformational changes to have possible functional implications, such as membrane interactions and the potential to act as molecular chaperone, as found in erythroid spectrin and cell signaling (Bhattacharyya et al., 2004; Machnicka, Grochowalska, Boguslawska, Sikorski, & Lecomte, 2012).

Estimation of the polarity of microenvironment fluorescence polarization and lifetime

Measurements of fluorescence polarization (anisotropy) are widely used in protein–ligand interactions, which could affect size, shape, and segmental flexibility of a macromolecule upon interaction with the fluorescent ligand, leading to change in polarization (anisotropy) (Chen & Bowman, 1965). An increase in the polarization (anisotropy) signifies that the molecule goes through more motionally restricted environment; this method is used to locate the possible position of the probe in the protein. In aqueous buffer, prodan shows polarization value of .03, which then increases to .28 and .29 upon addition of erythroid and nonerythroid spectrin, respectively (Supporting information Table S1). Similar increase in ANS polarization (Supporting information Table S1) was observed from .02 to .28 for binding of both the spectrin forms. The increase in fluorescence polarization implies that rotational motion of these probes is restricted in proteinous environment, and binding sites are hydrophobic in nature.

Fluorescence lifetime is one of the most sensitive parameter to have an idea on the microenvironment around the fluorophore, and also indicate the photophysics of the process. Prodan exhibits a bi-exponential decay with mean lifetime of 1.6 ns, but the decay profile of spectrin-bound prodan were best fitted with three exponentials (Supporting information Figure S3). A few representative decay profiles are shown in supporting Figure 3. Mean lifetime of prodan increases to 5.3 and 5.7 ns (Table 3), upon binding to erythroid and nonerythroid spectrin, respectively. Mean lifetime of ANS

Table 2. Thermodynamic parameters associated with the binding of prodan and pyrene with erythroid spectrin and nonerythroid spectrin at pH 7.8.

| Protein                  | ΔG (kcal/mol) | ΔH (kcal/mol) | ΔS (e.u.) |
|--------------------------|---------------|---------------|-----------|
| Erythroid spectrin       | -8.60 (-9.90) | .77 (-3.32)   | 31.46 (22.10) |
| Non-erythroid spectrin   | -9.21 (-10.72)| 8.232 (-5.31) | 58.53 (18.14) |

The values in the parenthesis indicate those in presence of pyrene.

Figure 3. Van’t Hoff’s plots of bindings of (A) prodan to erythroid and nonerythroid spectrin and (B) pyrene to erythroid and nonerythroid spectrin at different temperatures in buffer containing 10 mM Tris and 20 mM KCl at pH 7.8.
increases from .25 to 8.23 ns and 9.50 ns (Table 3), upon binding to erythroid and nonerythroid spectrin, respectively.

**Identification of prodan and pyrene binding site(s) in both erythroid and nonerythroid spectrin: a molecular docking study**

In order to find out the unique binding site(s) of both erythroid and nonerythroid spectrin at the atomic level, the molecular docking study was employed. Spectrin is a large heterodimeric molecule, consisting of α- and β-subunits. Both subunits assume flexible rod-like morphology from an extended array of triple α-helical repeat motifs consisting of about 107 amino acids. These helical repeat motifs, as a whole, constitute the rod domains of each subunit. One can structurally define spectrin as linear combinations of the actin-binding domain, which is formed by the C-terminal domain of α-spectrin with N-terminal domain of β-spectrin; the rod domain, containing the repeat motifs of the 3-helix bundle, and the self-association domain, which is formed by the N-terminal domain α-spectrin with the C-terminal domain of β-spectrin. The N-terminal domain of α-spectrin, the SH3 domain in the middle of the rod domain, the ankyrin binding of β-spectrin, and the self-associating domain of both erythroid and nonerythroid spectrin were used for docking study. We have compared the binding capabilities of the two hydrophobic ligands between the two forms of erythroid and nonerythroid spectrin. We have earlier shown that spectrin subunits bind prodan with lower affinity than intact spectrin (Bhattacharyya et al., 2004). Different structural domains of spectrin are

|                  | $\alpha_1$ | $\tau_1$ (ns) | $\alpha_2$ | $\tau_2$ (ns) | $\alpha_3$ | $\tau_3$ (ns) | Mean lifetime | $\chi^2$ |
|------------------|------------|---------------|------------|---------------|------------|---------------|--------------|---------|
| Prodans          | .58        | .69           | .41        | .204          | .16        | 1.60          | 1.20         |         |
| Spectrin bound prodans | .082       | 1.43          | .38        | 6.06          | .53        | .008          | 5.70         | 1.08    |
| Spectrin bound ANS | .18        | 2.54          | .84        | 8.46          | .04        | .07           | 8.23         | 1.11    |
| Fodrin bound Prodans | .12        | 1.42          | .44        | 5.55          | .42        | .431          | 5.30         | 1.20    |
| Fodrin bound ANS  | .18        | 3.45          | .76        | 1.07          | .05        | .18           | 9.50         | 1.15    |

*Lifetime of ANS .248 (taken from DeMatteis & Morrow, 2000).*

Figure 4. Energy minimized complexes of prodan and pyrene with ankyrin-binding domain of erythroid and nonerythroid spectrin. Prodans with ankyrin-binding domain of (A) erythroid spectrin and (B) nonerythroid spectrin and pyrene with ankyrin-binding domain of (C) erythroid spectrin and (D) nonerythroid spectrin.
created using Modweb Server (Table S2). One of the best results on docking studies between prodan and pyrene were obtained on the ankyrin-binding domain of spectrin, particularly in the nonerythroid form, as shown in Figure 4. Table 4 summarizes the binding energy between the ligands and the four different structural domains of erythroid and nonerythroid spectrins. Studies with the self-associating domain of spectrin also showed good docking of the ligands, particularly in the nonerythroid form, as shown in Figure 5 (Table 4). Results of the molecular modeling studies with the the SH3 domain and the N-terminal domain of α-spectrin, are shown in the Supporting Information Figure S4 and Figure S5, respectively. Negative binding energy and low inhibition constant are calculated using Autodock 4.2 software package, indicating favorable binding interaction between the ligands and the protein (Morris et al., 2009). Binding of prodan and pyrene are found to be favorable at the ankyrin-binding domain and the SH3 domain of both erythroid and nonerythroid spectrins. Comparison of hydrophobic ligand binding to the spectrins shows that nonerythroid spectrin binds tighter with lower binding energy than the erythroid counterpart. This also agrees well with our experimental result. The amino acid residues involved in the sphere of binding sites of prodan and pyrene are listed in Table 5. The molecular docking studies indicate that for the nonerythroid spectrin prodan, the molecule was adjacent to residues

Table 4. Computed binding energies of binding of four different domains of erythroid and nonerythroid spectrin.

| Spectrin  | Domain/model            | Prodan (interaction energy – kcal/mole) | Pyrene (interaction energy – kcal/mole) |
|----------|-------------------------|-----------------------------------------|-----------------------------------------|
| Erythroid| SH3 domain              | -3.42                                   | -4.33                                   |
|          | Self-association domain | -.56                                    | 1.89                                    |
|          | N-terminal domain, α-spectrin | -.66                               | 1.30                                    |
|          | Ankyrin binding domain  | -.95                                    | 2.78                                    |
| Non-erythroid| SH3 domain          | -3.86                                   | -4.66                                   |
|          | Self-association domain| -1.33                                   | -3.27                                   |
|          | N-terminal domain, α-spectrin | -1.22                               | -2.58                                   |
|          | Ankyrin binding domain  | -4.25                                   | -4.20                                   |

Figure 5. Energy minimized complexes of prodan and pyrene with the self-association domain of erythroid and nonerythroid spectrin. Prodan with self-association domain of (A) erythroid spectrin and (B) nonerythroid spectrin and pyrene with self-association domain of (C) erythroid spectrin and (D) nonerythroid spectrin.
Trp-40 and Ala-53, and the pyrene was adjacent to hydrophobic residues Leu-6, Ile-28, Leu-29 in SH3 domain, while in the ankyrin-binding domain, prodan is close to Leu-65, Val-72 Leu-75 and pyrene in vicinity of Ala-88, Leu-89. Similarly, in the SH3 domain of erythroid spectrin, prodan was in close contact with Pro-15, Val-18 and pyrene with Ala-12 and Pro-15, indicating mostly hydrophobic interaction between the ligand and the protein. It could be seen that the SH3 domain of both forms of spectrin could accommodate both the ligands and the protein. It could be seen that the SH3 domain of both forms of spectrin could accommodate both the ligands and the interaction between them, with the proteins being predominated by favorable contacts with hydrophobic amino acids. These observations provide a good structural basis to explain the blue shift of prodan emission maximum and two-fold increase of emission intensity, along with large increase in fluorescence polarization. In terms of ligand binding activities, SH3 domain is only implicated in the recognition of proline-rich peptides that involve conserved peptide-binding surface of SH3 (Saksela & Permi, 2012; Gushchina, Gabdulkhakov, Nikonov, & Filimonov, 2011). Moreover, as the hydrophobic interactions are non-directional and non-specific, it has been reported earlier that erythroid and nonerythroid spectrin bind prodan and pyrene with high affinity \( K_d = 0.02 - 0.50 \mu M \), and that the binding sites are strongly hydrophobic as indicated from the large 87 nm blue shift, and lifetime of spectrin bound prodan. The binding affinities of prodan to spectrins are much stronger, compared to other proteins reported so far, such as BSA, HSA, and tubulin. Weber and Ferris (1979) observed prodan, in water, give an emission maximum at 530 nm and by polarity measurement they showed that if all the prodan go to a hydrophobic core, then prodan emission is shifted to 400 – 410 nm. Later, Chong (1988) observed that in all membrane systems, prodan exhibits two local emission maxima at 510 and 435 nm. They explained that the 510 nm peak arises from prodan residing at the water–lipid interface and 435 nm emission peak arises from relocation of prodan molecule to less polar or hydrophobic region. Prodan in low concentration of spectrin gives two emission maxima, one at 520 nm and another at 430 nm, with intensity of the peak at 520 nm being higher than 430 nm. But, with increasing concentration of protein, the intensity of 430 nm peak increases, while the intensity of 520 nm decreases. This indicates relocation of prodan from polar region to less polar or hydrophobic region. Prodan molecule possesses a carbonyl and tertiary amine group and these two

| Protein                    | Domain                      | Ligand | Residue involved in binding the subset |
|----------------------------|-----------------------------|--------|---------------------------------------|
| Erythroid spectrin         | SH3 domain                  | Prodan | PRO (15), HIS (44), VAL (18), THR (19) |
|                            | Self-associating domain     | Prodan | PRO (15), THR (19), HIS (44)          |
|                            |                             | Pyrene | ALA (12), PRO (15), HIS (44)          |
|                            | N-terminal domain, α-spectrin| Prodan | PHE (115), SER (118), GLU (121)       |
|                            |                             | Pyrene | THR (27), GLU (28)                    |
|                            | Ankyrin binding domain      | Prodan | PHE (119), GLU (117)                  |
|                            |                             | Pyrene | LYS (67), GLU (71)                    |
| Non-erythroid Spectrin     | SH3 domain                  | Prodan | GLY (58), LEU (59), ASP (60)          |
|                            |                             | Pyrene | HIS (50), ARG (51), LEU (53)          |
|                            | Self-associating Domain     | Prodan | ASP (38), TRP (40), ALA (53)          |
|                            |                             | Pyrene | THR (2), LEU (6), ILE (28), LEU (29), THR (30) |
|                            | N-terminal domain, α-spectrin| Prodan | PHE (153), ALA (157)                 |
|                            |                             | Pyrene | SER (133), LEU (146)                  |
|                            | Ankyrin binding domain      | Prodan | LYS (20), PHE (20)                    |
|                            |                             | Pyrene | ILE (100), ASN (97)                   |
|                            |                             | Pyrene | ARG (53), LYS (57), ALA (88), LEU (89) |

**Table 5. Residues involved in binding with the hydrophobic ligands.**

- Trp-40 and Ala-53, and the pyrene was adjacent to hydrophobic residues Leu-6, Ile-28, Leu-29 in SH3 domain, while in the ankyrin-binding domain, prodan is close to Leu-65, Val-72 Leu-75 and pyrene in vicinity of Ala-88, Leu-89. Similarly, in the SH3 domain of erythroid spectrin, prodan was in close contact with Pro-15, Val-18 and pyrene with Ala-12 and Pro-15, indicating mostly hydrophobic interaction between the ligand and the protein.
- The observations provide a good structural basis to explain the blue shift of prodan emission maximum and two-fold increase of emission intensity, along with large increase in fluorescence polarization. In terms of ligand binding activities, SH3 domain is only implicated in the recognition of proline-rich peptides that involve conserved peptide-binding surface of SH3 (Saksela & Permi, 2012; Gushchina, Gabdulkhakov, Nikonov, & Filimonov, 2011). Moreover, as the hydrophobic interactions are non-directional and non-specific, it has been reported earlier that erythroid and nonerythroid spectrin bind prodan and pyrene with high affinity \( K_d = 0.02 - 0.50 \mu M \), and that the binding sites are strongly hydrophobic as indicated from the large 87 nm blue shift, and lifetime of spectrin bound prodan. The binding affinities of prodan to spectrins are much stronger, compared to other proteins reported so far, such as BSA, HSA, and tubulin. Weber and Ferris (1979) observed prodan, in water, give an emission maximum at 530 nm and by polarity measurement they showed that if all the prodan go to a hydrophobic core, then prodan emission is shifted to 400 – 410 nm. Later, Chong (1988) observed that in all membrane systems, prodan exhibits two local emission maxima at 510 and 435 nm. They explained that the 510 nm peak arises from prodan residing at the water–lipid interface and 435 nm emission peak arises from relocation of prodan molecule to less polar or hydrophobic region. Prodan in low concentration of spectrin gives two emission maxima, one at 520 nm and another at 430 nm, with intensity of the peak at 520 nm being higher than 430 nm. But, with increasing concentration of protein, the intensity of 430 nm peak increases, while the intensity of 520 nm decreases. This indicates relocation of prodan from polar region to less polar or hydrophobic region. Prodan molecule possesses a carbonyl and tertiary amine group and these two

**Hydrophobic interaction of ligand with erythroid and nonerythroid spectrin**

Spectrin has large number of hydrophobic patches through its polypeptide sequence to accommodate fatty acid and lipids, and these binding sites are in close proximity to tryptophan residue. The lipid binding sites of spectrin are grouped in \( \beta \)-spectrin in close proximity to the sites of attachment of ankyrin and 4.1, and later by comparative lipid binding study of erythroid and neural spectrin, An and coworkers reported that neuronal spectrin contains one extra lipid binding site at the N-terminal of the \( \alpha \)-chain (An et al., 2004; An, Guo, Wu, & Mohandas, 2004; An, Guo, Gratzer, & Mohandas, 2005; Wolny et al., 2011). It has been also shown earlier that erythroid spectrin can bind unrelated hydrophobic molecules, such as antimicrobial peptides, chromomycin and mithramycin (Majee et al., 1999), and the tertiary local anesthetic, dibucaine (Mandal & Chakrabarti, 2002). Our binding data here clearly indicates that both erythroid and nonerythroid spectrin bind prodan and pyrene with high affinity \( K_d = 0.02 - 0.50 \mu M \), and that the binding sites are strongly hydrophobic as indicated from the large 87 nm blue shift, and lifetime of spectrin bound prodan. The binding affinities of prodan to spectrins are much stronger, compared to other proteins reported so far, such as BSA, HSA, and tubulin. Weber and Ferris (1979) observed prodan, in water, give an emission maximum at 530 nm and by polarity measurement they showed that if all the prodan go to a hydrophobic core, then prodan emission is shifted to 400 – 410 nm. Later, Chong (1988) observed that in all membrane systems, prodan exhibits two local emission maxima at 510 and 435 nm. They explained that the 510 nm peak arises from prodan residing at the water–lipid interface and 435 nm emission peak arises from relocation of prodan molecule to less polar or hydrophobic region. Prodan in low concentration of spectrin gives two emission maxima, one at 520 nm and another at 430 nm, with intensity of the peak at 520 nm being higher than 430 nm. But, with increasing concentration of protein, the intensity of 430 nm peak increases, while the intensity of 520 nm decreases. This indicates relocation of prodan from polar region to less polar or hydrophobic region. Prodan molecule possesses a carbonyl and tertiary amine group and these two
functional groups reside at protein–water interface, resulting in emission at 520 nm. The 430 nm peak is likely to arise from prodan in an environment, which is hydrophobic. Our docking study indicates that some hydrophobic residues of the ankyrin-binding domain can provide such hydrophobic environment. While the naphthyl ring of prodan favors the hydrophobic pocket, the polar carbonyl and tertiary amine groups are stabilized by interactions with water and hydrophilic residues in contact Asp-60 and His-79 (Table 5). Comparative binding data indicates pyrene molecules binding to more hydrophobic sites of erythroid and nonerythroid spectrin than prodan. In case of pyrene molecules, absence of polar groups and one extra phenyl ring makes them more hydrophobic than prodan, which is corroborated from both the present experimental and theoretical study. The apparent dielectric constant for the hydrophobic binding site of erythroid and nonerythroid binding is in the range of 7–8.

Conclusion
We have compared the nature of binding of two hydrophobic ligands, prodan and pyrene, with two structurally homologous proteins, the erythroid and nonerythroid spectrin, using fluorescence spectroscopy and molecular modeling techniques. Prodan and pyrene bind specifically to both erythroid and nonerythroid spectrin with binding stoichiometry of 1 for both dimeric and tetrameric spectrin, indicating a unique binding site. Spectral changes of prodan and pyrene in protein appear to be a consequence of probe relocation. The physical origin of the difference in prodan and pyrene binding may be arising due to their structural differences i.e. prodan molecule remaining in a less hydrophobic region than pyrene molecule. Thermodynamic parameters indicate the binding to be entropy driven, however, with an appreciable enthalpic contribution in case of nonerythroid spectrin, revealing a classical enthalpy–entropy compensation mechanism. The hydrophobic sites in both forms of spectrin are of immense importance, since these sites could play key roles in mediating interactions with other proteins and membrane lipids. Molecular docking studies alone indicate that both prodan and pyrene strongly bind to all four major structural domains, the ankyrin-binding domain, the SH3 domain, the N-terminal domain of α-spectrin, and the self-association domain of both forms of spectrin. The experimental data, however, reveal the binding stoichiometry to be 1, particularly for the tetrameric nonerythroid spectrin and indicate toward a possible binding to the self-associating domain offering the common binding site. These interactions of membrane skeletal proteins could play crucial role in cell signaling, transport of small and large molecules across the membrane, membrane trafficking, and in the act of a molecular chaperone.

Supplementary material
The supplementary material for this paper is available online at http://dx.doi.10.1080/07391102.2013.793212.

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Abbreviations

- **ANS**: 1-anilinonaphthalene-8-napthalenesulfonic acid
- **Prodan**: 6-propinyl-2-(N, N-dimethyl-amino) naphthalene
- **PMSF**: phenyl methylsulfonyl fluoride
- **DTT**: dithiothreitol
- **EDTA**: ethylene diamine tetraacetic acid
- **EGTA**: ethylene glycol tetraacetic acid
- **SDS**: Sodium dodecyl sulfate
- **SH3**: Src homology domain 3
- **OPLS**: optimized potentials for liquid simulations

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