

# Polarity Estimate of the Hydrophobic Binding Sites in Erythroid Spectrin: A Study by Pyrene Fluorescence

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The apparent dielectric constant,  $\epsilon$ , of the hydrophobic pyrene binding sites in erythroid spectrin and human serum albumin (HSA) were estimated using the linear relationship [Turro, N.J., Kuo, P.L., Somasundaran, P. and Wong, K. (1986). *J. Phys. Chem.* 90, 288–291] between the ratio of the first (373 nm) and the third (384 nm) vibronic peak intensities ( $I_1/I_3$ ) and the dielectric constant of the bulk medium. Binding of the hydrophobic fluorescent probe, pyrene, to erythroid spectrin and HSA was determined from concentration dependent change in the ratio  $I_1/I_3$  from the emission spectra. Pyrene binds to spectrin ( $K_{app} = 6.2 \times 10^6 \text{ M}^{-1}$ ) with a higher affinity than that of HSA ( $K_{app} = 3.7 \times 10^4 \text{ M}^{-1}$ ) and the binding in both cases are saturable. The  $\epsilon$  for spectrin and HSA was estimated to be  $7 \pm 2.1$  and  $5.4 \pm 1.6$  respectively. A case study with spectrin, covalently labeled with pyrene maleimide, have been presented for aging of pyrene-labelled spectrin showing the potential of the use of vibrational peak ratios ( $I_1/I_3$ ) in the study of polarity of microenvironments in the neighborhood of cysteine residues of a protein. Large changes in the pyrene spectral components indicated conformational changes in the cysteine microenvironment of the protein upon storage at 4°C.

**KEY WORDS:** Hydrophobic sites; apparent dielectric constant; pyrene; erythroid spectrin.

## INTRODUCTION

Spectrin is the major constituent protein of the erythrocyte cytoskeleton that forms a filamentous network on the cytoplasmic face of the membrane. To establish the planar network, spectrin interacts with a large number of proteins like actin, adducin, ankyrin and protein 4.1 [1]. In addition to those proteins, spectrin binds to fatty acids and phospholipids [2–5]. Spectrin is a large dimeric, amphiphilic protein having hydrophobic stretches in its polypeptide sequence [6–8] that contain hydrophobic binding sites for fatty acids and lipids proximal to the

tryptophan residues in the protein [2,3]. Fluorescence quenching studies revealed binding of fatty acid derivatives [3] and other studies indicated binding of hydrophobic ligands like haemin and protoporphyrin to spectrin [9,10]. PRODAN (6-propionyl-2-(dimethylamino) naphthalene), a polarity sensitive hydrophobic fluorescent probe that exhibits large excited-state dipole moment and extensive solvent polarity-dependent shifts in the fluorescence emission maximum ( $\lambda_{max}$ ), binds erythroid spectrin with very high affinity [11]. Spectrin-bound PRODAN showed unique fluorescence with the largest blue-shift in the  $\lambda_{max}$  observed in any protein. We have further extended these studies on hydrophobic ligand binding to spectrin and have used pyrene, a widely used fluorescence probe to estimate the polarity of the hydrophobic binding sites in spectrin.

The fluorescence emission spectrum of monomeric pyrene is very sensitive towards solvent polarity [12,13]

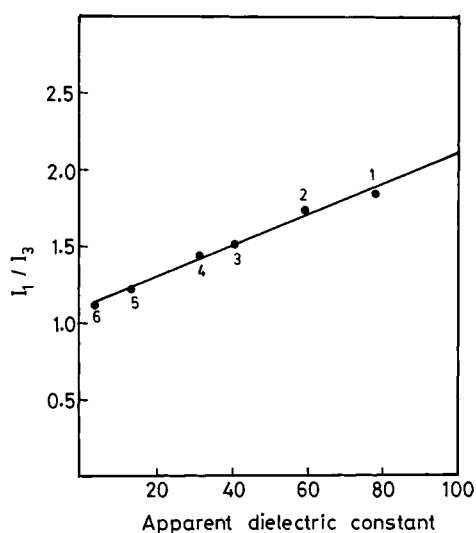
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and it is often used to estimate the polarity of micelles and membranes [14–18]. In apolar solvents the pyrene emission spectra is often split into five vibronic peaks at the wavelengths of 373 nm, 378 nm, 384 nm, 389 nm and 394 nm respectively. In polar and aqueous medium mostly three vibronic peaks are seen at 373 nm ( $I_1$ ), 384 nm ( $I_3$ ) and 394 nm ( $I_5$ ). In this work we have estimated the apparent dielectric constant of the pyrene-binding site in proteins e.g. erythroid spectrin and human serum albumin, for the first time, using the linear relationship between  $I_1/I_3$  and the apparent dielectric constant,  $\epsilon$ . A calibration curve was constructed between this ratio,  $I_1/I_3$  and the apparent dielectric constant,  $\epsilon$ , in the range between 3 (diethylether) and 78.5 (water), using various solvents (diethylether, methanol, ethanol, water and aqueous mixture of ethanol and methanol) as reported earlier by Turro and coworkers [19] from which the apparent dielectric constant of the pyrene binding sites in spectrin was determined (shown in Fig. 1). Human serum albumin (HSA), known to bind hydrophobic ligands, was also used for the present study [20,21]. Pyrene showed saturable binding with spectrin when the ratio  $I_1/I_3$  decreased with increasing concentration of the protein. The experimental values of the apparent dielectric constant of spectrin and HSA, estimated from the present study, are comparable to those used in theoretical calculations of electrostatic energies in proteins [22].

N-(1-Pyrenyl) maleimide is a thiol-directed fluorescent probe that has widely been used to study protein



**Fig. 1.** The calibration curve between the ratio,  $I_1/I_3$ , and the apparent dielectric constant,  $\epsilon$ , in the range between 3 (diethylether) and 78.5 (water), using various solvents. The points marked by numbers are quartz distilled water (1); methanol:water, 40:60, v/v (2); methanol:water, 80:20, v/v (3); methanol (4); ethanol (5); and diethylether (6).

structure and conformation [23,24]. Pyrene maleimide forms excimers after reaction with thiols of the cysteine residues in proteins e.g. tubulin, *E.coli* pyridine nucleotide transhydrogenase and lactose permease [23–25]. In a separate study with spectrin that is covalently modified with N-(1-pyrenyl) maleimide at the cysteine residues, we have also shown that the ratio,  $I_1/I_3$  changes upon long storage at 4°C without the formation of excimer emission bands.

## EXPERIMENTAL

Pyrene was obtained from Aldrich and was further purified over a silica gel column using cyclehexane as the solvent. N-(1-pyrenyl) maleimide, HSA and Tris were purchased from Sigma. The detailed procedure of purification of erythroid spectrin from ovine erythrocytes has been described earlier [26,27]. Concentrations of spectrin and HSA were determined using an absorbance of 10.7 and 5.3 at 280 nm for 1% spectrin and HSA, respectively, in a buffer containing 10 mM Tris-HCl, 50 mM NaCl, pH 7.4 [26,20]. Spectrin (1 mg/ml) was labeled with pyrene maleimide for 60 minutes at 25°C by adding 50-fold molar excess of pyrene maleimide in dimethylsulphoxide. The reaction was quenched by adding excess of 2-mercaptoethanol. The labeled protein was then separated from free pyrene maleimide by gel filtration on a Sephadex G-25 column [23]. The concentration of pyrene maleimide labeled spectrin (PM-spectrin) was determined by Lowry method [28] and that of pyrene was determined from the absorbance at 343 nm using a molar absorption coefficient of 42,000  $M^{-1}cm^{-1}$  for determining the labelling ratio in PM-spectrin. The labelling ratio of pyrene to spectrin was found to be 2 per spectrin dimer.

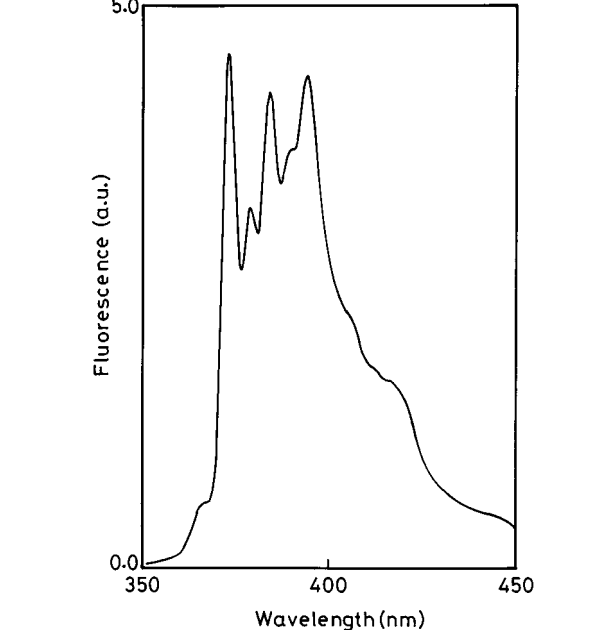
Steady state fluorescence was measured using a Hitachi F4010 spectrofluorometer using 1 cm pathlength quartz cell. Pyrene was excited at 335 nm and the emission spectra were recorded in the range of 350–450 nm. A bandpass of 1.5 nm for the excitation and 5 nm for emission slits was used to measure the fluorescence of pyrene. The buffer used in the present study contained 10 mM Tris-HCl and 50 mM NaCl, pH 7.4. For all experiments concentration of pyrene was kept at 0.2  $\mu$ M and the sample temperature was 25°C. For experiments with PM-spectrin excitation wavelength was at 343 nm.

## RESULTS

The ratio of the first (373 nm) and third (384 nm) vibronic peak intensities ( $I_1/I_3$ ) in pyrene emission spectra

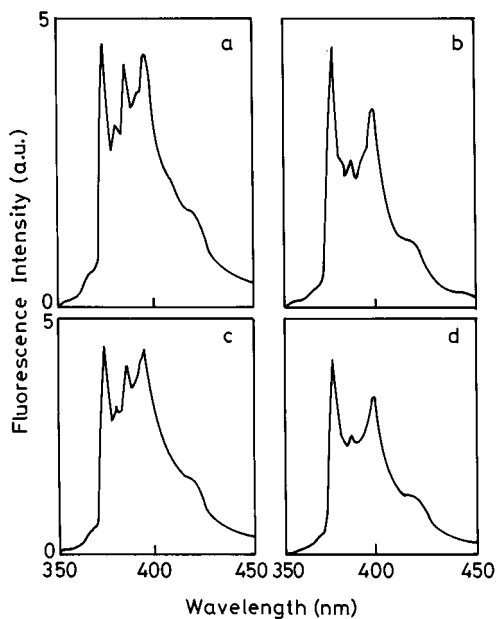
indicates the apparent polarity of the solvent. A lower value corresponds to nonpolar ( $I_1/I_3 = 0.6$  for cyclohexane), and a higher value to polar ( $I_1/I_3 = 1.84$  for water), solvents respectively [29]. The calibration line between the ratio ( $I_1/I_3$ ) and the apparent dielectric constant is shown in Fig. 1 that is used in estimating the dielectric constants of the hydrophobic binding sites in spectrin and HSA. Pyrene monomeric emission spectra in two different concentrations of the two proteins are shown in Fig. 2. It has been observed that the vibronic fine structures of pyrene monomer in high concentrations of HSA and spectrin are well defined without any change in the spectral shape (Figs. 2a & 2c). From this well-defined spectral nature the ratio,  $I_1/I_3$  could be evaluated at different concentrations of the proteins and hence the apparent binding constant of the two proteins. Figure 3 shows the emission spectrum of HSA-bound pyrene in presence of 80  $\mu\text{M}$  HSA where five vibronic peaks at the wavelengths of 373 nm, 378 nm, 384 nm, 389 nm and 394 nm respectively are well-defined.

The concentration dependence of  $I_1/I_3$  of pyrene fluorescence in spectrin and HSA are shown in Fig 4. The ratio,  $I_1/I_3$  decreases with increasing concentration of the proteins before it reaches saturation at 450 nM spectrin and 50  $\mu\text{M}$  HSA without any change on further increase in protein concentrations. At low concentrations of proteins, pyrene mostly remain in aqueous environment and at a higher protein concentration, all pyrene molecules

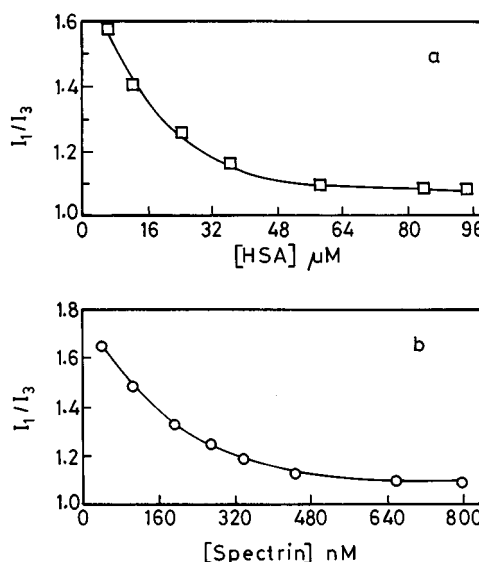


**Fig. 3.** The fluorescence emission spectra of 0.2  $\mu\text{M}$  pyrene at 25°C in the presence of 80  $\mu\text{M}$  HSA clearly indicating the five vibronic peaks at the respective wavelengths of 373 nm, 378 nm, 384 nm, 389 nm, and 394 nm.

were bound to the hydrophobic sites of the proteins. The apparent binding constants for the molecular complexes of pyrene-spectrin and pyrene-HSA were determined from the plot of  $I_1/I_3$  against concentration of the proteins



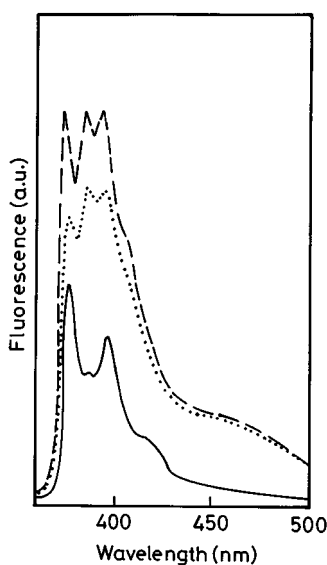
**Fig. 2.** The fluorescence emission spectra of 0.2  $\mu\text{M}$  pyrene at 25°C in (a) 85  $\mu\text{M}$  HSA; (b) 1  $\mu\text{M}$  HSA; (c) 500 nM spectrin; (d) 10 nM spectrin.



**Fig. 4.** Plot of the ratio ( $I_1/I_3$ ) of pyrene fluorescence as a function of increasing protein concentrations: (a) for HSA and (b) for spectrin. The solid line is the theoretical best fit to the data points. Pyrene concentration is 0.2  $\mu\text{M}$ .

(Fig. 4). The affinity constants were estimated to be  $6.2 \times 10^6 \text{ M}^{-1}$  for spectrin and  $3.7 \times 10^4 \text{ M}^{-1}$  for HSA respectively, using a simple model assuming a stoichiometry of 1:1 between the proteins and the pyrene, shown earlier in PRODAN binding to spectrin [11]. From such binding isotherms of pyrene with both proteins the  $I_1/I_3$  values were chosen from saturating concentrations of the proteins at or beyond which the  $I_1/I_3$  values remain unchanged (Fig. 4). The apparent dielectric constant of the hydrophobic pyrene binding sites were then estimated using this ratio of  $I_1/I_3$  from the calibration line shown in Fig. 1. The  $\epsilon$  for spectrin and HSA were estimated to  $7 \pm 2.1$  and  $5.4 \pm 1.6$  respectively. We have also estimated the  $\epsilon$  of pyrene binding sites in bovine serum albumin to be 14 from the reported value of  $I_1/I_3$  (1.18) of 1% BSA (150  $\mu\text{M}$ ) solution [30].

At low protein concentrations the pyrene emission spectrum looked identical to that of the PM-spectrin, only three of the vibronics,  $I_1$ ,  $I_3$  and  $I_5$  at 373 nm, 384 nm and 394 nm respectively are well defined, shown in Figs. 2d, 2b, and 5. We have also shown in a separate study that the ratio  $I_1/I_3$  can be a sensitive index in studying the structural changes in proteins that are covalently labelled by pyrene maleimide. The change in the three vibronic peak intensities and the ratio  $I_1/I_3$  in PM-spectrin upon long storage at  $4^\circ\text{C}$  revealed changes in the polarity of the local environment experienced by the free sulphhy-



**Fig. 5.** Fluorescence emission spectra of PM-spectrin, with 2 pyrene per spectrin dimen, stored at  $4^\circ\text{C}$  for a period of 30 days. The spectra are not drawn on the same ordinate scale. The peak intensities of  $I_1$  (373 nm) and  $I_3$  (384 nm) are 78.7 and 47.4 for both freshly labelled and 1 day old PM-spectrin (solid line), 42.6 and 40.2 for 15 day old PM-spectrin (dashed line), and 33.2 and 35.5 for 30 days old PM-spectrin (dotted line), respectively.

dryl groups in spectrin. Fig. 5 shows visible changes in the fine structure of monomeric pyrene emission spectrum in PM-spectrin that was stored at  $4^\circ\text{C}$  over a period of 15 days during which the pyrene label does not come out in the aqueous phase by dissociation from the -SH group. The ratio  $I_1/I_3$  evaluated from the pyrene emission spectra of PM-spectrin changes drastically in course of 15 days. In other proteins e.g. tubulin and *E.coli* pyridine nucleotide transhydrogenase, excimer emissions were observed [23,24]. We have not observed any excimer fluorescence even in the PM-spectrin with labeling ratio of 20 indicating a large distance between each pyrene molecule i.e. the cysteine residues in the polypeptide backbone of spectrin.

## DISCUSSION

The vibrational peak ratios ( $I_1/I_3$ ) of pyrene emission have been used for the estimation of apparent dielectrics in detergent micelles and membranes by many other groups [12–19]. However, the present report is the first of its kind where the apparent dielectric constant of the hydrophobic pyrene binding site in proteins has been estimated. The apparent dielectric constant estimated for the hydrophobic binding sites in erythroid spectrin and serum albumins are in the range of 4–9 (Table I). An apparent dielectric constant of 2–5 is commonly used in the calculation of electrostatic energies of proteins [22]. The general argument given for such an assumption is that protein having a semirigid interior has dipoles oriented in specific directions. However, it has also been reported that the  $\text{pK}_a$  shifts computed for ionizable groups that are not actually in contact with a charged ligand will be rather accurate when an  $\epsilon$  of 20 is used [31]. In order to arrive at a reliable value, experimental determination of the  $\epsilon$ , of protein interior is important. The present work provides with a simple fluorescence spectroscopic method to estimate the apparent dielectric constant of the

**Table I.** Apparent Dielectric Constant Determined from Pyrene Fluorescence Measurements

Protein	Dielectric constant ( $\epsilon$ )
Spectrin <sup>a</sup>	$7.0 \pm 2.1$
HSA <sup>a</sup>	$5.4 \pm 1.6$
BSA <sup>b</sup>	14.0

<sup>a</sup> The apparent dielectric constant was determined in 10 mM Tris-HCl, 50 mM NaCl, pH 7.4.

<sup>b</sup> The  $I_1/I_3$  value in 0.2M phosphate buffer, pH 5.6, was taken from Ref. 30 and  $\epsilon$  was evaluated from the calibration shown in Ref. 19.

interior of proteins that shows saturable binding with pyrene.

The hydrophobic binding sites in spectrin is not well characterized. However, the hydrophobic binding site in spectrin is of extreme importance in the context of its interaction with the membrane components e.g. lipids, fatty acids, and other hydrophobic ligands. It has been earlier shown that completely unrelated hydrophobic ligand molecules such as antitumor antibiotics, chromomycin and mithramycin could bind spectrin [27]. The previous study on the interaction of the fluorescent probe, PRODAN with spectrin indicated that the polarity of the PRODAN binding site in spectrin is like that of chlorobenzene as estimated by comparing the wavelength of maximum emission [11,32]. PRODAN and one of its analog, DANCA has been successfully used as hydrophobic marker to estimate polarity of the heme-binding pocket in apomyoglobin [33]. There is another report for apomyoglobin where the emission energy of DANCA indicated a polarity of the heme binding pocket to be similar to that of dimethylformamide [34]. Human serum albumin has been subjected to hundreds of ligand binding studies. Most ligands are bound to HSA reversibly with typical association constants ranging from  $10^4$  to  $10^6$  M<sup>-1</sup> [35]. In serum albumin it is generally recognized that there are a small number of distinct binding locations for hydrophobic ligands. All these aspects in the structure of serum albumins have been reviewed by Carter & Ho [35]. The importance of hydrophobic binding sites in proteins has been best established in molecular chaperones. These proteins have a domain containing hydrophobic amino acid residues located at the surface of the protein enabling them to bind lipophilic substances such as ANS or bis-ANS [36,37].

The data on spectrin that was labelled at the cysteine residues using the probe, pyrene maleimide have been presented as a case study for aging of pyrene-labelled spectrin (PM-spectrin) showing the potential of the use of vibrational peak ratios ( $I_1/I_3$ ) in the study of polarity of microenvironments in the neighborhood of cysteine residues of a protein. The apparent dielectric constant have been observed to change from 62 (freshly labelled spectrin,  $I_1/I_3 = 1.67$ ) to less than 5 (15 days old spectrin at 4°C,  $I_1/I_3 = 1.06$ ) upon storage of pyrene maleimide labelled spectrin at 4°C. Pyrene maleimide has been covalently attached to the cysteine residues, the location of which is completely different from that of free pyrene which binds to a hydrophobic binding site of the two proteins. PM-spectrin showed changes in the vibronic structures in upon storage (Fig. 5) at 4°C without showing any excimer emission. The ratio  $I_1/I_3$  in PM-spectrin decreased with longer storage time at 4°C and indicated

that the pyrene moiety gets into more nonpolar environment upon storage.

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