Fluorescence quenching of spectrin and other red cell membrane cytoskeletal proteins

Relation to hydrophobic binding sites

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The intrinsic fluorescence of spectrin is strongly quenched by low concentrations of 2-bromostearate. This results from binding at a series of hydrophobic sites. Analysis of dynamic fluorescence quenching by acrylamide, iodide and caesium ions, separately and in conjunction with 2-bromostearate, leads to the conclusion that most of the tryptophan side-chains are exposed to solvent. The sites at which the fatty-acid-quenched tryptophans are located apparently interact with the lipid bilayer in the cell, as judged by quenching by bromostearate dissolved in the lipid phase. A minor proportion of the side-chains in native spectrin give rise to sharp proton magnetic resonance signals, indicative of segmental mobility; these chain elements contain some tryptophan residues, as revealed by weak downfield signals from the heterocyclic ring protons. These signals are not appreciably perturbed by stearic acid or by phosphatidylserine liposomes, suggesting that the hydrophobic binding sites are not in mobile chain elements. By contrast with a series of globular proteins which, with the exception of serum albumins, show little or no quenching by 2-bromostearate, the peripheral red cell membrane skeletal proteins ankyrin (and its spectrin-binding domain), protein 4.1 and (to a lesser extent) actin show evidence of a high affinity for the hydrophobic ligand and may, like spectrin, interact directly with the bilayer *in situ*.

INTRODUCTION

The protein network that covers the cytoplasmic surface of the red cell membrane, and on which the elastic properties of the membrane depend, is attached to the bilayer through well-defined interactions with intrinsic membrane proteins. The stability and elasticity of the membrane are a function primarily of the surface concentration of protein (in particular of the major component, spectrin) in contact with the phospholipid bilayer (Shields *et al.*, 1987; Waugh & Agre, 1988). There is abundant evidence in favour of a weak but globally important interaction between spectrin and phosphatidylserine (Mombers *et al.*, 1980; Cohen *et al.*, 1986; Maksymiw *et al.*, 1987). It has also been found (Isenberg *et al.*, 1981; Bonnet & Begard, 1984; Sikorski *et al.*, 1987; Streichman *et al.*, 1991) that spectrin contains a large number of hydrophobic sites for the attachment of fatty acids and lipids, and many or all of these sites evidently contain, or are close to, tryptophan side-chains (Isenberg *et al.*, 1981; Streichman *et al.*, 1991). These residues are of particular interest, for most of the tryptophan content is accounted for by two positions in the 106-residue repeating unit (Speicher & Marchesi, 1984) of spectrin and related proteins, at one of which it is totally and at the other largely conserved. This makes the interaction of spectrin with hydrophobic ligands particularly amenable to study by fluorescence methods.

Another membrane skeletal constituent, protein 4.1, also appears to interact in a functionally important way with phosphatidylserine (Sato & Ohnishi, 1983; Rybicki *et al.*, 1988; Cohen *et al.*, 1988; Shiffer *et al.*, 1988), a lipid confined in the normal red cell to the inner membrane leaflet. Actin has also been reported to bind to liposomes if divalent cations are present (St-Onge & Gicquard, 1990). Thus it appears possible that all the major membrane skeletal constituents may interact directly with the lipid bilayer.

We present here the results of a study of the properties and environments of the hydrophobic binding sites of spectrin and of

its associated proteins, primarily by fluorescence quenching techniques.

MATERIALS AND METHODS

Proteins were prepared from human red cells by established procedures. Spectrin dimer was extracted from the membranes at low ionic strength at 35 °C and purified by chromatography on Sepharose 4B (Gratzer, 1981). Ankyrin was prepared by the procedure described by Pinder *et al.* (1989) and protein 4.1 by dissociation of isolated membrane skeletons in 2 M-Tris, followed by gel filtration in the same medium (Ohanian & Gratzer, 1984), containing in addition 0.1% Tween 20. The detergent was eliminated from the purified protein by chromatography on DEAE-cellulose in an f.p.l.c. instrument. The column was equilibrated with 20 mM-Tris, pH 8.0, and after application of the sample was washed in the same buffer to elute detergent. The protein was eluted at 0.18 M in a 0–0.4 M-NaCl gradient in 20 mM-Tris, pH 8.0. That the column indeed retains the protein, while allowing complete removal of detergent, was shown by monitoring the separation of added Triton X-100, which can be readily detected by its ultraviolet absorption spectrum. Actin from rabbit skeletal muscle was prepared by the method of Spudich & Watt (1971). Protein concentrations were determined spectrophotometrically. Phosphatidylserine liposomes were generated by sonication of dried films in buffer. White ghosts and inside-out membrane vesicles, depleted of extrinsic proteins, were prepared by standard methods (Bennett & Stenbuck, 1980b).

2-Bromostearic acid was a gift from Dr. N. M. Green (National Institute for Medical Research, London, U.K.) and 9(10)-bromostearic acid was from Sigma. Acrylamide was from FSA. Attempts were made to decrease the u.v. absorption by recrystallization from chloroform and by sublimation. These procedures were found to cause little further reduction in the absorbance at 295 nm, which represents the edge of the intrinsic absorption band of the compound.

Fluorescence was measured in a Perkin–Elmer–Hitachi

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MPF 3L fluorimeter, with fluorophore absorbances at the excitation wavelength of not more than 0.02. Quenchers were added from stock solutions, and corrections were made for dilution; in the case of the bromostearates ethanol was used as the solvent, and the volume of ethanol added was kept below 5%. Corrections were also applied for Rayleigh and Raman scattering, using a buffer blank, and for the inner filter effect. For the latter, calculated correction factors, which have often been used, proved to be unreliable (cf. Parker, 1968). We accordingly constructed a correction curve by adding to the protein solution an inert chromophore with no fluorescence at room temperature and with a similar change of absorbance with wavelength in the critical region to that of acrylamide; guanosine 5'-monophosphate fulfilled these conditions. No inner filter correction was required for the brominated fatty acids.

For proton magnetic resonance spectroscopy the protein was dialysed with four changes against 10 ml volumes of 0.1 M-NaCl/20 mM-sodium phosphate in $^2\text{H}_2\text{O}$. Spectra were obtained at 20 °C at a protein concentration of about 2 mg·ml⁻¹ in a Bruker 250 MHz instrument with a solvent presaturation pulse. In general about 5000 pulses were accumulated.

RESULTS

As shown previously (Isenberg *et al.*, 1981), 2-bromostearate, and to a lesser extent stearic acids, brominated at positions (9 and 10 or 17 and 18) remote from the carboxyl group, are strong quenchers of the intrinsic fluorescence of spectrin at low concentrations that imply site binding. This is also shown by the disappearance of scattering from solutions to which the poorly soluble bromostearate has been added when spectrin is present. At a 2-bromostearate concentration of 20 μM , *N*-acetyltryptophanamide and the equivalent tyrosine derivative, as well as ribonuclease, ovalbumin, trypsinogen and β -lactoglobulin, showed no or minimal quenching (0–7%), and lysozyme was quenched by 20%. Human and bovine serum albumins, which have very high binding capacities for hydrophobic ligands, especially long-chain fatty acids (Goodman, 1958), by contrast showed about 65% quenching.

Fig. 1 shows the dependence of the quenching profiles for 2-bromostearate as a function of spectrin concentration. To relate these curves to binding isotherms requires the assumption that each bound ligand quenches the fluorescence to the same extent. This may be a plausible approximation if all sites are close to the conserved tryptophan residues and quench their emission totally, so that the unquenched fraction (some 20%) represents the unperturbed emission from residues not in the hydrophobic sites. At all events, the curves at all concentrations can be quite well fitted with a single association constant of $(0.5\text{--}1) \times 10^6 \text{ M}^{-1}$ for 650 identical sites per spectrin dimer, that is to say about 17 for each structural repeating unit. These and other bromostearate binding data are summarized in Table 1.

To obtain more information about the environments of the different tryptophan populations, dynamic quenching experiments were performed. The neutral and anionic quenchers, acrylamide and iodide ion, gave linear Stern–Volmer plots. In terms of the modified Stern–Volmer equation of Eftink & Ghiron (1976), i.e.:

$$F_0 F = (1 + K_Q [Q]) \cdot \exp V[Q] \quad (1)$$

where F is the fluorescent intensity at a quencher concentration $[Q]$, F_0 the unperturbed intensity, K_Q the collisional quenching constant and V a measure of the probability of static complex formation, this implies that there is no ground state interaction with the quencher ($V = 0$). The quenching rate constant (k_q) is given by K_Q/τ_0 , where τ_0 is the unquenched excited state lifetime, and is a measure of the degree of exposure of the fluorophores.

This can thus in principle be expressed (Bukulova-Orlova *et al.*, 1974) as $(K_{Q,\text{protein}}/K_{Q,\text{tryptophan}}) \cdot (q_{\text{tryptophan}}/q_{\text{protein}})$, where the q values are the quantum yields for the protein and free tryptophan derivatives. In practice, the quenching rate constants for exposed tryptophans in denatured proteins are usually much smaller than for free tryptophan derivatives or small peptides (Ivkova *et al.*, 1971; Eftink & Ghiron, 1976; Divita *et al.*, 1991). Results for spectrin are given in Table 2, from which it can be seen that the tryptophan side-chains are moderately accessible to acrylamide, i.e. within the range found in most globular proteins (Eftink & Ghiron, 1976), but the accessibility to iodide ions appears considerably larger, which suggests that the fluorophores are predominantly in cationic environments. Moreover, since there

Fig. 1. Quenching of spectrin fluorescence by 2-bromostearate

The points show the fluorescence intensity (F) relative to the unperturbed value (F_0) at 338 nm for spectrin dimer concentrations of 15 nM (●), 60 nM (■) and 90 nM (▲). The two curves are calculated for spectrin concentrations of 15 and 90 nM, with the assumption of 640 sites, all with the association constant $0.8 \times 10^6 \text{ M}^{-1}$, and all causing equal quenching. Inset: Low-field n.m.r. spectra at 250 MHz, showing tryptophan signals of native spectrin (a); the same with added stearic acid (b); and the same as (a), with added phosphatidylserine liposomes (c). The sharp signal in (c) is due to an impurity in the phospholipid.

Table 1. Binding of 2-bromostearate to membrane skeletal proteins

The fractional quenching of fluorescence at saturation is given by $\Delta F/F_0$; n is the number of fatty-acid-binding sites (from the inflection point), and K_{app} is the apparent association constant, assuming identical quenching by all bound ligand molecules. nd, not determined.

Protein	$\Delta F/F_0$	n	$K_{\text{app.}} (\text{M}^{-1})$
Spectrin	0.8	650	5×10^5
Protein 4.1	0.4	45	$> 10^{6*}$
Ankyrin	0.65–0.7	300	1×10^6
72 kDa fragment†	0.7	90	$2_s \times 10^6$
F-actin	0.45	35	2×10^5
Ghosts	0.55	nd	nd
IOV‡	0.5	nd	nd
Proteolysed IOV§	0.5	nd	nd

* Curve cannot be fitted on the basis of the assumption of identical quenching.

† Chymotryptic ankyrin fragment, containing spectrin-binding site.

‡ Inside-out vesicles (IOV) after extraction of spectrin and actin.

§ Vesicles, as above, treated with chymotrypsin to eliminate all but intramembrane domains of integral membrane proteins.

Table 2. Dynamic quenching of spectrin fluorescence

K_Q is the Stern–Volmer quenching constant measured at the emission maximum (338 nm); excitation at 295 nm. The quenching of residual fluorescence, unaffected by 2-bromostearate, measured at the plateau bromostearate concentration (200 μM), is indicated by 'after 2-BS'. For comparison, the following are literature data for acrylamide quenching constants: *N*-acetyltryptophanamide, 17.5; glucagon, 10.5; globular proteins, 1–9 (Eftink & Ghiron, 1976). The quenching constant for *N*-acetyltryptophanamide by iodide is 6.0 (Divita *et al.*, 1991); and the quenching constant for tryptophan by caesium ion is 2.1 (Ivkova *et al.*, 1971).

Quencher	K_Q (M^{-1})	Stern–Volmer profile
Acrylamide	4.2	Linear
after 2-BS	3.2	$f_a = 0.75^*$
Iodide ion	3.5	Linear
after 2-BS	0.5	Linear
Caesium ion	0.8*	$f_a = 0.15^\dagger$; $K_Q = 8^\dagger$
after 2-BS	0	—
Trichloroethanol	~ 20	Shows upward curvature

* Determined from slope of Stern–Volmer plot at the origin.

† Fraction of exposed tryptophan residues, assuming that the remainder are totally inaccessible to the quencher; f_a is obtained from eqn. (2) (Lehrer, 1971), which gives a value for the quenching constant of the exposed fraction.

Fig. 2. Fluorescence emission spectra of unperturbed spectrin (a), spectrin in the presence of 200 μM -2-bromostearate (b) and the latter after further addition of 0.3 M-acrylamide (c)

The excitation wavelength was 295 nm. The spectra are uncorrected, and intensities are in arbitrary units.

is no upward curvature of the Stern–Volmer plots, any buried tryptophans, totally inaccessible on the time-scale of the excited state lifetime, must comprise a small proportion of the total. The quenching constants are wavelength-dependent, and, as expected, are larger the longer the wavelength of measurement, because of the red-shifted emission of the fluorophores in the polar aqueous medium.

The very low degree of quenching by caesium ions is consistent with the inference of a cationic environment for the tryptophans. The Stern–Volmer plots also show strong downward curvature, indicative of heterogeneity of quenching. If one makes the assumption that there is one class of identically accessible fluorophores and the remainder are completely shielded, the relation of Lehrer (1971) applies:

$$F_0/\Delta F = (f_a K_Q [Q])^{-1} + f_a^{-1} \quad (2)$$

in which f_a is the fraction of solvent-accessible fluorophores and $\Delta F = F_0 - F$. This yields a linear plot for quenching by caesium ion, with values for the fraction of masked residues of between 0.9 and 0.75, depending on the wavelength. The corresponding quenching constants, which are in the range 5–10 M^{-1} , are however anomalously high for caesium ion.

We have also used the highly hydrophobic quencher 2,2,2-trichloroethanol (Eftink *et al.*, 1977). This proved to be highly efficient and yielded Stern–Volmer plots with pronounced upward curvature, reflecting ground-state interaction. Such systems can be analysed by eqn. (1) (Eftink & Ghiron, 1976) or more directly by taking the fluorophores engaged in a ground-state complex to be fully quenched, with only the free fraction susceptible to dynamic quenching. Then, for a ground-state bimolecular association constant K and a quencher concentration $[Q]$ in large excess over that of fluorophores, the fraction of free fluorophores is $1/(1 + K[Q])$. This leads to the modified Stern–Volmer equation:

$$F_0/F = (1 + K[Q]) \cdot (1 + K_Q[Q])$$

which rearranges to:

$$\Delta F/F[Q] = K + K_Q + KK_Q[Q] \quad (3)$$

so that a plot of $\Delta F/F[Q]$ against $[Q]$ gives K and K_Q from the slope and intercept respectively. At 380 nm a linear plot is obtained, but at the fluorescence maximum there is upward curvature, reflecting possibly unfolding of the protein by the quencher. A ground-state association constant of 3–5 M^{-1} and a quenching constant of 10–13 M^{-1} can be extracted from the data. The latter is of a similar order to those found for proteins with hydrophobic binding sites, such as serum albumins (Eftink *et al.*, 1977).

The above results suggest that the hydrophobically sited tryptophans are substantially exposed to the solvent. To examine this further, dynamic quenching studies were carried out on spectrin that had been quenched to the plateau level by 200 μM -2-bromostearate. The residual emission spectrum under these circumstances (Fig. 2), amounting to some 10% of the integrated unquenched intensity, displayed a markedly blue-shifted maximum (327 nm), characteristic of shielded fluorophores (Burstein *et al.*, 1973). Iodide ions did not measurably quench the fluorescence at the maximum, and at longer wavelengths showed low quenching to a plateau level (Fig. 3). Acrylamide gave appreciable quenching with a downward curvature; analysis by eqn. (2) gave a fractional exposure of the side-chains responsible for the residual fluorescence of about 0.75 and a quenching constant of about 3 M^{-1} . This implies that the acrylamide can penetrate into hydrophobic regions of the structure (Eftink & Ghiron, 1976).

That the majority of the tryptophans are accessible to the solvent was also shown by examining the perturbation of their absorption spectra by deuterium oxide. Exchange of the heterocyclic N–H hydrogen for deuterium generates a small spectroscopic shift, measurable as a difference spectrum (Nakanishi *et al.*, 1978). If tryptophan side-chains are shielded from the solvent, the kinetics of the perturbation are slow. In the case of spectrin the difference spectrum was fully developed within the time of mixing (~ 10 s) and no further exchange occurred over a period of hours. Thus all or most of the tryptophans are indeed in contact with the solvent.

The proton magnetic resonance spectrum of spectrin revealed

Fig. 3. Stern-Volmer plots for quenching of spectrin fluorescence by iodide ions

Closed symbols refer to unliganded spectrin, with emission at 380 (●), 338 (■) and 320 (▲) nm. Open symbols refer to quenching by iodide at the corresponding wavelengths of the residual fluorescence of spectrin (30 nm) in the presence of 200 μ M-2-bromostearate.

that a minor part of the structure is highly segmentally mobile (Calvert *et al.*, 1980; Fung *et al.*, 1986). The sharp resonances that lead to this conclusion have a small contribution in the downfield region from tryptophan ring protons (Fig. 1, inset). We have examined the effect on this part of the spectrum of added stearic acid and phosphatidylserine liposomes. As can be seen, these do not appreciably perturb the ring proton signals. Thus the likelihood is that the motile polypeptide chain elements do not contain the hydrophobic binding sites, in which by inference a large proportion of the tryptophans, in particular the conserved fraction, are located.

We have studied bromostearate binding to the remaining major membrane skeletal proteins. Protein 4.1 shows strong quenching by bromostearate with profiles indicative of rather strong interactions, as for spectrin (Fig. 4). Saturation occurs at a mole ratio of about 45 fatty acids/protein. However, in this case the assumption of identical quenching by all bound ligand molecules does not give a satisfactory fit to the data. From the ligand concentration at which a plateau is reached one can nevertheless infer that all of the fatty acid molecules that bind do so with association constants of not less than about 10^6 M^{-1} .

For acrylamide, the Stern-Volmer plot shows downward curvature. Assuming no static component, analysis by eqn. (2) leads to a quenching constant at the emission maximum of $19 M^{-1}$, but this may again reflect penetration of the acrylamide into the hydrophobic interior; the quenching constant and the proportion of exposed residues decrease somewhat with increasing wavelength of measurement. The unperturbed emission spectrum has a maximum at 330 nm, characteristic of shielded fluorophores (Burstein *et al.*, 1973), and a perceptible shoulder at about 350 nm, due to a solvent-exposed population. These distinct classes of tryptophans may pertain to the independent functional domains of the protein (Correas *et al.*, 1986). There is further efficient quenching by acrylamide of the residual fluorescence in the presence of a saturating concentration of 2-bromostearate.

Fig. 4 also shows the 2-bromostearate quenching of ankyrin fluorescence, and here also there is evidence of saturable binding, though with quenching of only some 45% of the tryptophan emission. Saturation corresponds to about 300 molecules of fatty

Fig. 4. Fluorescence quenching of red cell membrane skeletal proteins in solution by 2-bromostearate

(a) Ankyrin (■) and the spectrin-binding domain of ankyrin (●); protein concentrations were 0.053 μ M and 0.33 μ M respectively. The curves are calculated with the arbitrary assumption of identical sites, all giving rise to the same degree of quenching, and correspond to 340 sites per molecule with an association constant of $1 \times 10^6 M^{-1}$ for ankyrin, and 90 sites per molecule with an association constant of $1.25 \times 10^6 M^{-1}$ for the fragment. (b) F-actin (●) and protein 4.1 (■); protein concentrations were 0.74 μ M and 0.26 μ M respectively. The curve for actin is calculated using the same assumptions as above, with 35 sites per molecule and an association constant of $2 \times 10^5 M^{-1}$. The data for protein 4.1 could not be fitted satisfactorily with the same assumptions.

acid per molecule of protein. The profiles can be arbitrarily fitted with a single association constant of $1 \times 10^6 M^{-1}$. In this case, quenching by the 9(10)-isomer is almost as great, indicating that the sites in this instance are not markedly anionic, as they are in spectrin. Moreover, examination of the spectrin-binding domain of ankyrin, i.e. the 72000- M_r tryptic fragment (Bennett & Stenbuck, 1980a) revealed a comparable degree of quenching, with an inflection at about 90 mol/mol, showing that the binding sites are distributed between the spectrin-binding and the other domains.

F-actin gave definite but weaker quenching by 2-bromostearate (approx. 40%, with an apparent association constant for 35 nominally identical sites of $2 \times 10^5 M^{-1}$) and by 9(10)-bromostearate (approx. 20%).

Quenching of protein fluorescence by bromostearate is totally suppressed by low concentrations of non-ionic detergent. This

means that the quencher partitions overwhelmingly into the micellar phase. The same is true for the red cell membrane. Spectrin was added, in the presence and in the absence of 2-bromostearate, to inside-out vesicles, from which endogenous spectrin, actin, ankyrin and 4.1 had been extracted by successive incubations with media of low and high ionic strength. (The spectrin concentration was $20 \mu\text{g} \cdot \text{ml}^{-1}$ and the vesicle concentration corresponded to 0.3 mg of total protein $\cdot \text{ml}^{-1}$.) The vesicles were pelleted and the fluorescence of the spectrin in the supernatant was measured. The intensities were identical in the two cases, showing that the quencher had partitioned entirely into the vesicles.

Haemoglobin-free ghosts were accordingly examined and their fluorescence in suspension in iso-osmotic buffer was found to be quenched by 2-bromostearate to the extent of about 55 % (Table 1). The membrane vesicles resulting from extraction of spectrin and actin gave a similar degree of quenching. Since spectrin comprises some 25 % of the total membrane protein, it follows that its fluorescence is quenched similarly *in situ* and in solution, and that quenching of the tryptophan residues occurs from within the bilayer. When the vesicles were treated with chymotrypsin to eliminate ankyrin, 4.1 and the cytoplasmic *N*-terminal domain of band 3 (Steck, 1978), more than 50 % quenching was again seen. Thus it appears that a large proportion of the peripheral membrane skeletal proteins interact directly with the bilayer to allow quenching by bromostearate dissolved in the lipid.

DISCUSSION

Our results indicate that bromostearates detect hydrophobic patches on the surface of proteins. Quenching of fluorescence occurs as a result of site-binding of the fatty acid, rather than by a collisional or short-range interaction, such as has been inferred to occur with dibrominated hydrophobic quenchers (Bolen & Holloway, 1990). For spectrin, 9,10-dibromostearate and 17,18-dibromostearate are in fact much weaker quenchers than 2-bromostearate (Isenberg *et al.*, 1981). The hydrophobic sites in spectrin must be distributed fairly uniformly along the polypeptide chains, and are presumably a characteristic of the individual 106-residue repeating units. The fold, which is inferred to contain a three-stranded α -helical coiled-coil element (Speicher & Marchesi, 1984; Cross *et al.*, 1990), evidently brings anionic groups into apposition with the hydrophobic binding site. This is shown by the dependence of quenching efficiency on the proximity of the carboxylate group to the heavy atom in the bromostearates, as well as the large disparity in the efficiencies of the anionic and cationic collisional quenchers relative to their effects on small uncharged fluorophores. A similar situation evidently prevails in serum albumin (Ivkova *et al.*, 1971). Moreover, Lehrer (1971) has shown that quenching by iodide ions of tryptophans in copolymers with lysine and glutamic acid is characterized by quenching constants of 22 and 0.3 M^{-1} respectively at ionic strength 0.18 M (at which the value for *N*-acetyltryptophanamide is 12 M^{-1}).

The tryptophan residues in spectrin are largely exposed to the solvent, and the evidence suggests that they are involved in the direct binding of the protein to the lipid bilayer, which, though undoubtedly weak when assessed in terms of interactions with liposomes (Bitbol *et al.*, 1989), should be co-operative for the cytoskeletal network on the membrane and must certainly be functionally important (see e.g. Maksymiwi *et al.*, 1987). If most of the tryptophans are in the lipid-binding sites, as indicated by the extent of quenching, these must include the conserved residues at positions 12 and especially 45 (Speicher & Marchesi, 1984). The tryptophans in the segmentally mobile chain elements,

revealed by the n.m.r. spectra, form a small proportion of the total tryptophan content, as judged by the very weak signal, and our results strongly suggest (although they do not prove) that these parts of the chain do not engage in hydrophobic interactions.

We cannot of course conclusively relate the numerous fatty-acid-binding sites in spectrin to its interaction with the membrane. Nevertheless, spectrin undoubtedly binds to lipid, and no other function for abundant and rather strong binding of hydrophobic ligands by the protein in the cell can readily be envisaged. Moreover, the fatty-acid-binding sites do appear to interact with the bilayer, in that there is quenching of the tryptophan fluorescence by the membrane-soluble bromostearate.

Whereas quantitative estimates of numbers of tryptophans subject to quenching must be treated with caution because of the possibility that quenched fluorophores may act as energy sinks for close-lying indoles, this is less likely to be significant for spectrin because of the large separation between structural elements (the end-to-end distance of the spectrin dimer in solution is approx. 500 nm). The estimations of affinities of bromostearate for the hydrophobic sites must be regarded as no more than semi-quantitative, depending as they do on the implicit assumption that all bound ligands give the same degree of quenching, and the fit to a single binding process is not taken to imply that this is a physically valid model.

Our data suggest that the possession of abundant hydrophobic binding sites may be a general property of peripheral membrane proteins, judged at least by the components of the red cell membrane skeleton, including ankyrin, protein 4.1 and, to a lesser extent, actin.

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