BINDING OF HYDROPHOBIC LIGANDS TO SPECTRIN

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1. Introduction

Spectrin is the major protein of the red cell membrane cytoskeleton, a network, which covers the inner surface of the membrane and is responsible for maintaining the shape, stability and mechanical properties of the cell. The primary site of attachment of the cytoskeleton to the membrane is by way of an association between spectrin and a receptor protein, 2.1 or ankyrin [1,2], which is itself bound to the preponderant transmembrane protein, band 3 [3,4]. The mechanism by which the cytoskeleton acts to control the membrane properties is not clear; some evidence has been adduced in favour of a direct interaction between spectrin and the lipid bilayer, and it has been suggested [5,6] that this interaction stabilises the membrane and preserves the asymmetric distribution of the phospholipids between the inner and outer leaflet [7]. No direct binding studies with dispersed phospholipids have been carried out, probably due to the technical problems that such measurements present, but there are several reports [8–13] of perturbations in the properties of phospholipid vesicles, (all, however, by crude spectrin, contaminated with other cytoskeletal proteins) and one [14] of a relatively high propensity of spectrin to penetrate phospholipid monolayers. The observed effects have been reported to be greatest with phosphatidylserine [6,14], which is a component present exclusively in the inner leaflet of the membrane [7].

Spectrin, in terms of its amino acid composition and indeed its solubility properties, is by no means a particularly hydrophobic protein. For example the 'hydrophobicity index', based on the free energies of transfer of the amino acid side chains from water to an organic solvent [15], is 880, which is similar to that of myosin and considerably lower than for example those of actin, serum albumin and more especially of known integral membrane proteins. At the same time the technique of charge-shift electrophoresis, using mixed detergent species [16], suggests that the structure of spectrin may embody a degree of amphiphilicity [17]. Proton magnetic resonance, moreover, has shown that part of the protein is highly flexible in character, and markedly hydrophobic in composition [18]. We show here, using a new group of hydrophobic model ligands which are strong quenchers of intrinsic protein fluorescence, that spectrin contains a large number of binding sites for hydrocarbon chains, and infer the presence of intermittent hydrophobic regions along the molecule.

2. Materials and methods

Spectrin was prepared in the dimeric form by extraction of ghosts at low ionic strength at 35°C for 10 min. In all other details the procedure was as in [19]. The crude spectrin was purified by chromatography on Sepharose 4B and the dimer and oligomer were recovered, the latter containing also the cytoskeletal proteins, actin and 4.1. Samples were screened for purity by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Protein concentrations were determined spectrophotometrically, taking the specific absorptivity $E^{1\%}_{1\,cm}$ (280 nm) = 10.7 [20]. Fluorescence measurements were performed with a Perkin-Elmer Hitachi MPF-3L spectrofluorimeter. The solvent was 0.1 M NaCl, 20 mM Tris (pH 8.0) except for the experiments at low ionic strength, and kept at 15°C. Protein solutions of absorbance 0.007–0.02 at the excitation wavelength (280 nm) were titrated with bromostearate or its homologues, dissolved in ethanol. The final ethanol concentration did not exceed 5% (v/v).

The quenching agents used in this study were 2-bromostearic acid and 9,10- and 17,18-dibromostearic acid. The latter were prepared by bromination with bromine in ether [21] of octadec-9-enoic (oleic) acid and octadec-17-enoic acid. The last was synthesised by Dr F. D. Gunstone [22] and the others were commercial samples.

The brominated fatty acids are somewhat photochemically labile. Care was therefore taken to reduce the exposure of the solutions to ultraviolet light by minimising the entrance slit (which was generally 2 nm) and the time of irradiation. Emission intensity was measured at 330 nm. For competition experiments, lysolecithins of different chain lengths (Sigma) were either added at fixed quencher concentration, or titrations with the quenchers were performed at fixed lysolecithin concentration. Egg phosphatidylcholine was added when required after measurement of the fluorescent intensity in the presence of varying concentrations of 2-bromostearate. Critical micelle concentrations for the lysolecithins were checked by measurement of the change in fluorescent intensity of 1-anilino-8-naphthalene sulphonate with increasing lysolecithin concentrations [23].

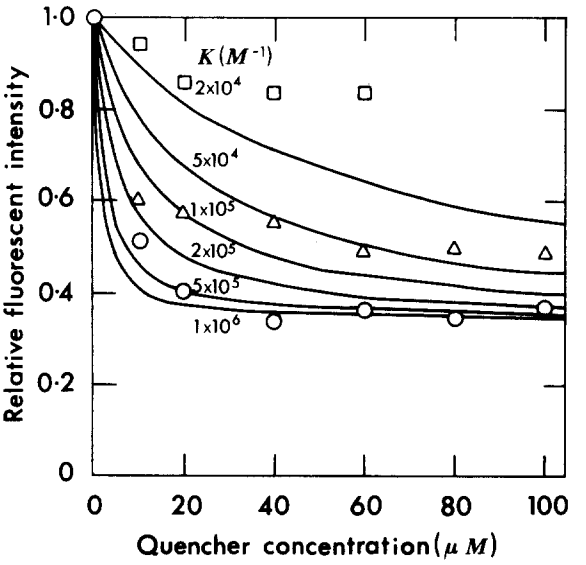

Fig.1. Fluorescence quenching curves for bromostearate binding to spectrin dimers. Protein was 6.2 μg/ml and the solvent 0.1 M NaCl, 20 mM Tris (pH 8.0). The curves are arbitrary binding profiles for 100 independent sites for 2-bromostearate/dimer, each giving the same proportional degree of quenching, with the equilibrium constants indicated; (○) corresponding experimental points. 17,18-Dibromostearate (□); 9,10-dibromostearate (△).

3. Results

2-Bromostearate was found to quench the intrinsic (tryptophan) fluorescence of spectrin with high efficiency. Because of the low (sub-mM) concentration range within which the effect was observed, the process is characterised as complex formation and not dynamic quenching. The concentration of the ligand throughout was below the self-association limit, and the temperature was well below the Krafft point. We cannot rule out the possibility that the solutions were supersaturated, but except where noted there was no detectable aggregation or precipitation: centrifugation of the solution did not diminish the measured fluorescence, neither was there any significant change in the Rayleigh scattering intensity when the excitation and emission monochromators were set at the same wavelength. The non-aqueous solvent at the concentration added to the protein had no effect. A typical binding profile is shown in fig.1. This curve cannot be fitted in terms of one or a small number of very strong sites. In any case since the fluorescence comes from ~70 tryptophan residues (determined spectrophotometrically in terms of the tryptophan: tyrosine ratio [24]) and some 60% of the fluorescence is quenched, such a model would scarcely be feasible. The system is too complex to justify any attempt at a detailed analysis of the number of sites and their different affinities. However, as fig.1 indicates, a model involving a large number of comparable binding sites of moderate affinity (say 100 sites/dimer with association constants of the order of 10^5–10^6 M^{-1}) gives an adequate fit of the data.

The binding is clearly predominantly hydrophobic because a 10-fold higher concentration of the shorter chain homologue, 2-bromomyristate, is required for comparable quenching, 2-bromopalmitate giving an intermediate effect, and 2-bromolaurate some 2 orders of magnitude lower quenching at the same concentration. On the other hand at low ionic strength (5 mM NaCl, 1 mM Tris) there is perceptibly greater quenching, suggesting either a change in orientation of the bound ligand molecules or an increase in the number of binding sites. This may be due to the well-documented expansion of the molecule at low ionic strength [25,26] or to an increased degree of electrostatic interaction with the ligands. The quenching induced

by the 17,18-dibromostearate was an order of magnitude lower than that by 2-bromostearate at the same concentration, the 9,10-isomer giving an intermediate effect. With increasing concentration of the 17,18-isomer there was a perceptible rise in Rayleigh scattering, due apparently to the formation of aggregates. To establish whether the lower quenching is due to a diminished efficiency of excitation deactivation or to weaker binding, competition experiments were performed. It was found that when equimolar amounts of 2-bromostearate and 17,18-dibromostearate were present the quenching was intermediate between the levels recorded with the 2 ligands separately, though closer to that induced by 2-bromostearate. Thus the binding of 2-bromostearate is stronger than that of 17,18-dibromostearate but that when bound it is also a more efficient quencher. It may be inferred that the ends of the hydrocarbon chains bearing the carboxyl group are on average closer to tryptophan side chains than are the ω-ends.

Binding is substantially and rapidly reversible, for on addition of phosphatidylcholine vesicles the fluorescence returns to nearly its unperturbed value. It may be presumed that the bromostearate molecules are partitioning into the vesicles. However, we cannot rule out a degree of competition for sites on the spectrin. Phosphatidylserine vesicles have a similar effect. Attempts to achieve competition with monomeric phospholipids did not give conclusive results because of the very low (e.g., 0.5 nM for egg phosphatidylcholine [27]) critical micelle concentration of the phospholipid. Competition experiments were attempted with lauryllysophosphatidylcholine, which has a critical micelle concentration of 0.2 mM [28], working below this level. No significant competition was observed, showing that this molecule does not bind effectively at the hydrophobic sites. This may be partly a consequence of its relatively polar nature, but probably more particularly of the shorter chain length, which would rule out effective competition against the quencher at attainable monomer concentrations.

We note finally that the quenching profiles obtained for the oligomeric complex of spectrin, actin and 4.1 were not significantly different from those for spectrin alone. This indicates that there is no significant occlusion of binding sites by the other proteins, which is in accord with expectation, since association occurs only at the ends of the elongated spectrin molecules. The weight proportion of spectrin in the complex is large [29], so that binding to actin or 4.1 would probably escape detection.

4. Discussion

The brominated fatty acids are clearly effective quenchers of indole fluorescence. They compete with fatty acids at hydrophobic binding sites. Bromostearate for example binds to bovine serum albumin with $K_a \geqslant 10^5\ M^{-1}$ (N. M. G., unpublished).

The presence of a large number of hydrophobic binding sites on the spectrin molecule follows from the shape of the binding curves and also from the high degree of quenching: in a molecule 100 nm long [30], containing 70 tryptophan residues, that can collectively be quenched to ⩾60%, unless all the tryptophans are clustered in a short segment of the chain the binding sites must be distributed along much of the total span. The location of bromines at the end of the chain remote from the polar group evidently not only reduces the efficiency of quenching by the bound ligand but also reduces its affinity for the binding sites. This may be due to the increase in dipolar character of the hydrocarbon, or more probably to some form of steric obstruction. The intermediate result obtained when the bromine atoms are in the middle of the chain suggests that much of the free energy of binding comes from the attachment of the part of the chain near the ω-end.

There is no justification for assuming that all sites are equivalent in affinity and structure, but a further argument in favour of spaced hydrophobic regions comes from proteolytic digestion experiments, which suggest the presence of a structural regularity in the molecule [31]. It may be noted that similarly abundant binding of a very different pair of ligands, haemin and protoporphyrin IX, to spectrin has been observed [32,33]. The segmentally flexible, hydrophobic portion of the molecule giving rise to a sharp-line proton magnetic resonance spectrum [18] must thus be supposed to comprise a considerable number of short stretches of polypeptide chain, possibly those at which proteolysis most readily occurs to give a progression of fragments [31]. If these all constitute relatively weak sites of interaction with lipid, at which the chain can penetrate into the bilayer, such a disposition could more readily explain how the network of filamentous molecules over the membrane surface might have a large effect on the stability of

the bilayer as has been suggested [5,6], while at the same time dissociating rather readily under conditions in which the primary binding interaction with protein 2.1, or ankyrin is weakened [1,2].

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