Biochimica et Biophysica Acta, 443 (1976) 198–205

BBA 77396

LIPID-PROTEIN RELATIONSHIPS IN ERYTHROCYTE MEMBRANES REVEALED BY PARAMAGNETIC QUENCHING OF PROTEIN FLUORESCENCE

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(Received January 13th, 1976)

SUMMARY

1. Paramagnetic quenching of erythrocyte membrane protein fluorescence by nitroxide-labelled lipid analogues has been studied as a function of temperature and quencher concentration, as well as after cross-linking of membrane proteins by glutaraldehyde.

2. Quenching due to nitroxide stearates reveals a static component, due to binding of quencher molecules to protein, superimposed upon a diffusion-limited component.

3. Static quenching decreases progressively above 35 °C, a temperature region where a thermotropic discontinuity is known to occur (Bieri, V. G. and Wallach, D. F. H. (1975) Biochim. Biophys. Acta 406, 415–423).

4. Diffusion-limited quenching becomes progressively more prominent as the temperature is raised above 15 °C.

5. Exposure of membranes to varying concentrations of glutaraldehyde indicates that membrane proteins relatively poorly accessible to cross-linking are those responsible for the membrane thermotropism above 35 °C.

6. Protein fluorophores accessible to androstane nitroxide are saturated at a low quencher/protein ratio. This ratio is stable below 35 °C but increases by 50% between 35 and 55 °C.

INTRODUCTION

In previous publications we have introduced the use of paramagnetic fluorescence quenching as a new fluorescence probe technique for the monitoring of fluorophore-lipid proximity relationships in artificial [1] and biological membranes [2–4]. We have demonstrated that the paramagnetic quenching of fluorescence sensitively

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reflects the known thermotropic properties dipalmitoyllecithin and dipalmitoyllecithin-cholesterol liposomes [1]. Using different classes of paramagnetic lipid derivatives we have also detected segregation of sterol from membrane protein in erythrocyte ghosts and thymocyte plasma membranes [3, 4]. Finally, we have shown that lipid-protein interactions in erythrocyte membranes can change with temperature and pH in physiological ranges [3].

In this paper we use paramagnetic quenching of tryptophan fluorescence to study alterations of lipid-protein and lipid-lipid interactions in erythrocyte membranes between 5 and 45 °C as a function of quencher concentration. We further report on the effect of mild glutaraldehyde fixation on the thermotropism between 35 and 45 °C previously detected by paramagnetic quenching studies and we also attempt to establish a correlation between nitroxide-induced lysis of intact erythrocytes and paramagnetic quenching in erythrocyte membranes.

MATERIALS AND METHODS

2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-nitroxide stearate), 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (16-nitroxide stearate) and 17-β-hydroxy-4',4'dimethylspiro-[15α-androstan-3,2'-oxazolidin]-3'-yloxyl (androstane nitroxide) were purchased from Syva (Palo Alto, Calif.) and dissolved in methanol (Fisher certified) at 10^{-2} M. Glutaraldehyde (50 %, w/w, Fisher biological grade) was diluted with phosphate-buffered saline, pH 7.4 (GIBCo) to specified concentrations; other chemicals were of analytical grade. Human erythrocyte membranes were prepared from freshly drawn heparinized blood (O, Rh^-) as in ref. 5 and were suspended at a concentration of 35 μg protein/ml in 50 mM phosphate, 1 mM NaN_3, pH 7.4, for quenching experiments. Paramagnetic quenching of protein fluorescence was measured as described previously [3] except that replicate individual samples were incubated for 60 min for each quencher concentration instead of titrating replicate single samples.

For glutaraldehyde treatment, packed erythrocyte ghosts (750 μg protein) were suspended in 8 ml 60 mM phosphate, pH 7.4, 0, 0.05, 0.2 and 0.5 mM in glutaraldehyde. After 60 min at 4 °C the reaction mixtures were dialyzed against 50 mM phosphate, 1 mM glycine, pH 7.4, for 16 h. Aliquots of the dialysates were diluted with buffer to 35 μg protein/ml and used for quenching experiments as in ref. 3. Other aliquots were used for dodecyl sulfate polyacrylamide electrophoresis as in ref. 6.

For hemolysis experiments $1.7 \cdot 10^7$ washed erythrocytes were incubated at temperatures ranging from 5 to 45 °C in 3 ml hypotonic NaCl, 10 mM phosphate, pH 7.4, $1 \cdot 10^{-7}$–$2.4 \cdot 10^{-5}$ M in 5-nitroxide stearate. The tonicity of the saline-phosphate solution was adjusted to always yield 50 % lysis (rel. hemolysis = 1) at the test temperature in the absence of nitroxide stearate. After 30 min, unlysed cells were spun down and relative hemolysis determined as previously described [7].

RESULTS

Temperature and concentration dependence of fluorescence quenching

The suppression of membrane protein fluorescence by 5-nitroxide stearate is

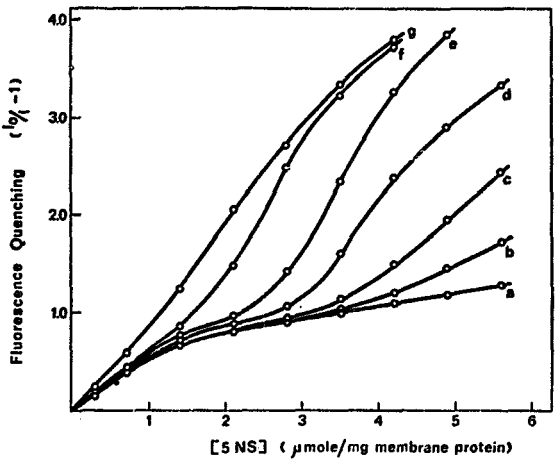

Fig. 1. Temperature dependence of protein fluorescence quenching in erythrocyte membranes by 5-nitroxide stearate. a, 5–15 °C; b, 20 °C; c, 25 °C; d, 30 °C; e, 35 °C; f, 40 °C, and g, 45 °C.

illustrated in Fig. 1 for temperatures ranging from 5 to 45 °C. The quenching curves are identical up to 15 °C and exhibit a saturation component up to approx. 2 μmol nitroxide stearate/mg protein, followed by a linear segment. Between 15 and 25 °C this relationship becomes biphasic: below 2 μmol/mg the quenching curve is as at 15 °C or below but at higher concentrations (> 3 μmol/mg protein) there is a second increment in quenching. This multiphasic pattern becomes more and more prominent between 25 and 35 °C, but diminishes again at the highest temperature tested (45 °C), where the quenching curve approaches a straight line between 0 and 3 μmol/mg.

A similar pattern is seen with 16-nitroxide stearate, except that the quenching efficiency is only 40 % that of 5-nitroxide stearate throughout. Moreover, the onset of the linear segment at low temperatures and the beginning of the second quenching phase above 15 °C occur at about 3 μmol 16-nitroxide stearate/mg protein.

In contrast to the stearate derivatives, androstane nitroxide exhibits quenching curves with saturation characteristics at all temperatures up to 55 °C (Fig. 2) but a much lower quenching efficiency at all concentrations. However, whereas the quenching curves are essentially invariant between 5 and 35 °C, quenching efficiency increases by approx. 50 % at all quencher concentrations between 35 and 55 °C.

Influence of glutaraldehyde cross-linking

Cross-linking of erythrocyte proteins with glutaraldehyde at bulk concentration up to 0.05 mM does not significantly influence the shapes of the quenching curves observed with 5-nitroxide stearate, although the quenching efficiency is reduced. At 0.2 and 0.5 mM glutaraldehyde the extent of quenching drops further and the thermotropic discontinuity between 35 and 55 °C is abolished (Fig. 3).

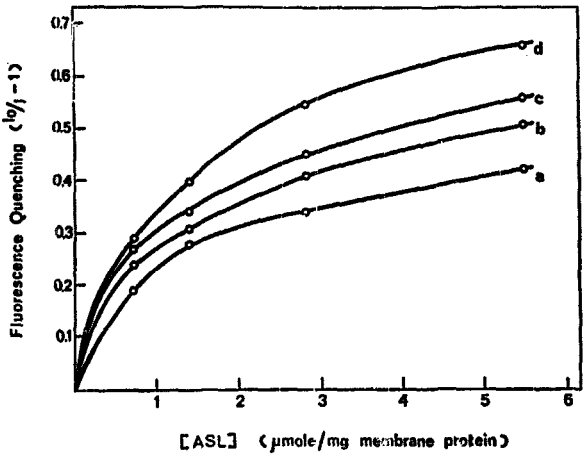

Fig. 2. Temperature dependence of protein fluorescence quenching by androstane nitroxide. a, 5–35 °C; b, 40 °C; c, 45 °C, d, 55 °C.

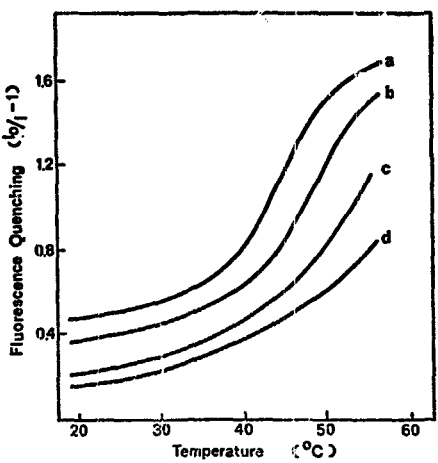

Fig. 3. Temperature dependence of 5-nitroxide stearate-induced fluorescence quenching in glutaraldehyde-treated erythrocyte membranes. Glutaraldehyde: a, 0; b, 0.05 mM; c, 0.2 mM; and d, 0.5 mM.

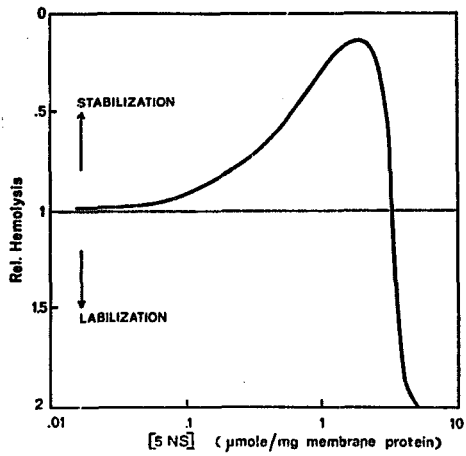

Fig. 4. 5-Nitroxide stearate-induced stabilization – labilization sequence of osmotically stressed erythrocytes. Osmolarities adjusted (Table I) to give 50 % hemolysis without 5-nitroxide stearate. Curves obtained as in ref. 7 and lie within 5 % for all points at all temperatures.

Electrophoretic analyses reveal that, up to 0.05 mM glutaraldehyde the protein bands 1, 2, and 4.1 [6] are decreased selectively, leading to accumulation of highly aggregated material at the tops of the gels. Higher glutaraldehyde levels lead to cross-linking of all membrane protein components. These results are in accord with prior observations by Steck [8].

As shown before [7] and in Fig. 4, 5-nitroxide stearate at low concentrations stabilizes osmotically stressed erythrocytes, but at high concentrations induces cell lysis (even without osmotic stress). We find that the curves recorded between 5 and 45 °C yield identical patterns. Maximal stabilization occurs at 10^{-5} M 5-nitroxide stearate (approx. 2 μmol/mg protein) and complete hemolysis develops at slightly higher concentrations. However, the osmotic stress required to produce 50 % lysis in the absence of nitroxide varies approximately linearly with temperature, ranging from 75 mM NaCl at 5 °C to 61 mM at 45 °C (Table I).

TABLE I

EFFECT OF TEMPERATURE ON THE CONCENTRATION OF NaCl SOLUTIONS GIVING 50 % HEMOLYSIS

Temperature (°C)	[NaCl] at 50 % lysis (mM)
5	75
15	71
25	67.5
35	64
45	61

DISCUSSION

Paramagnetic quenching of protein fluorescence by nitroxide-labelled lipids measures the accessibility of tryptophan and tyrosine residues to these molecules [1, 3, 4]. In simple solution systems fluorescence quenching processes are usually dynamic, i.e. diffusion limited, with the corresponding Stern-Volmer plots [9] exhibiting a linear relationship between the extent of quenching and quencher concentration. However, in more complex systems static quenching may also occur due to close long-term associations between quencher and fluorophore [9, 10] and we have demonstrated [2–4] that in certain membranes both static and dynamic quenching can occur simultaneously. This situation is further complicated by the fact that many nitroxide-labelled lipid analogues tend to partition preferentially into fluid lipid domains [11], as well as by the circumstance that some of these probes tend to perturb their immediate environment (e.g. refs. 12–14). To clarify these processes, we have monitored the quenching of erythrocyte membrane tryptophan fluorescence by 5-nitroxide stearate and androstane nitroxide as a function of temperature and quencher concentration.

We find that 5-nitroxide yields identical relationships between quenching and quencher concentration at all temperatures between 5 and 15 °C. All of the curves indicate static quenching, saturated at approx. 2 μmol quencher/mg membrane protein, superimposed upon a dynamic, diffusion-controlled component (linear segment, Fig. 1a). Indeed, the non-linear segment due to static quenching does not vary significantly with temperature up to 35 °C (Fig. 1b and c), but it tends to disappear above 40 °C (Fig. 1f and g). We have previously excluded apparent quenching due to spectral shifts secondary to formation of ground-state complexes [1]. Because the interaction distance in paramagnetic quenching of fluorescence lies near 4–6 Å [15], the static component is thus most reasonably ascribed to binding of quencher molecules near tryptophan and/or tyrosine residues. The progressive reduction of the static component above 35 °C, despite higher total quenching, indicates less nitroxide binding at the higher temperatures. It corresponds to the thermotropic quenching discontinuity reported previously [1] and can be attributed to an alteration in membrane protein structure.

According to quenching theory [9, 10] the dynamic component can be ascribed to 5-nitroxide stearate molecules that collide randomly with protein fluorophores in diffusion-limited encounters. Two processes might be involved: (a) direct contact of protein fluorophores with "fluid" lipid, (b) exchange of quencher molecules between a protein-associated lipid boundary layer and a bulk lipid phase.

The fact that the dynamic quenching component is stable between 5 and 15 °C is compatible with our Raman analyses indicating no significant variation of acyl chain packing in this temperature range, but a well-defined lipid state transition between 14 and 10 °C [16]. However, the deviations that occur above 20 °C are highly complex and the increased quenching observed cannot be attributed simply to an increasing collision rate between quenchers and fluorophores, although this is indubitably involved. Indeed curves Fig. 1d–f suggest appearance of new binding sites (inflections between 1.5 and 3.5 μmol/mg protein), as well as increased dynamic quenching. It is conceivable that the 5 °C–22 °C transition observed by Raman spectroscopy [16] is one that allows greater partition of nitroxide stearate into mem-

branes and possibly even allows greater membrane perturbation by the nitroxides.

To test the last possibility we have examined the effect of various concentrations of 5-nitroxide stearate on osmotically stressed erythrocytes [7] at varying temperatures. We found that providing the tonicity was always adjusted to give 50% hemolysis at the temperature under test in the absence of nitroxide, increasing levels of nitroxide stearate always protected identically against osmotic lysis regardless of temperature, with maximum stabilization always occurring at 10^{-5} M nitroxide (2 μmol/mg protein) and lysis developing at slightly higher concentrations.

These results, taken together with our quenching data indicating static quenching, i.e. binding of quencher to protein, below 2 μmol/mg protein, support the proposals that the stabilization phase involves alterations of membrane protein [7, 17] and that lysis is a "detergent effect" (e.g. refs. 7 and 17).

We used cross-linking with glutaraldehyde to provide some clues as to what membrane proteins might be involved in the thermal discontinuities in paramagnetic fluorescence quenching above 37 °C, noted here and previously [1]. Monitoring the effects of glutaraldehyde by dodecyl sulfate polyacrylamide electrophoresis, we find results identical to those of Steck [8], namely components of bands 1, 2 and 4.1 are readily cross-linked at much lower glutaraldehyde levels than other membrane proteins, e.g. band 3, the principal hydrophobic protein [18]. Importantly, the membrane protein thermotropism is not abolished under conditions where bands 1, 2 and 4.1 are preferentially cross-linked, implying that the thermal effects observed concern the other membrane proteins.

Fluorescence quenching with androstane nitroxide (Fig. 2) is not temperature sensitive up to 35 °C. Also, the saturation character and the low quenching efficiency at all temperatures tested support our previous finding that this probe has only limited contact with membrane proteins, perhaps because it distributes largely into cholesterol-rich membrane domains [3, 4].

Our experimentation with nitroxide-labelled lipid derivatives has revealed that these probe molecules can report on important aspects of lipid-protein interaction in some biomembranes. At low levels, stearate nitroxides reflect primarily static protein-lipid interactions, whereas high probe concentration also monitors alterations in the lipid matrix. These facts, as well as present and earlier data, suggesting partitioning of androstane nitroxide into cholesterol-rich membrane domains, indicate that one cannot assume statistical distribution of probe molecules in biomembranes.

ACKNOWLEDGEMENTS

Supported by awards from the National Cancer Institute (CB 43922) and from the American Cancer Society (D.F.H.W.).

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