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VARIATIONS OF LIPID-PROTEIN INTERACTIONS IN ERYTHROCYTE GHOSTS AS A FUNCTION OF TEMPERATURE AND pH IN PHYSIOLOGICAL AND NON-PHYSIOLOGICAL RANGES

A STUDY USING PARAMAGNETIC QUENCHING OF PROTEIN FLUORESCENCE BY NITROXIDE LIPID ANALOGUES

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SUMMARY

1. Incorporation of stearic acid nitroxides into erythrocyte ghosts markedly depresses the fluorescence of membrane protein tryptophan residues. 5-Nitroxide stearate quenches fluorescence more efficiently than 16-nitroxide stearate. Both compounds exhibit dynamic (diffusion-limited) quenching above 0.28 μmol/mg protein and static quenching at lower nitroxide protein ratios. Static quenching can be attributed to high affinity binding of nitroxide stearates by membrane protein. The dynamic phase represents distribution of the stearate analogues into a fluid lipid system.

2. Protein fluorophores accessible to quenching by a cholesterol analogue, androstane nitroxide, are saturated at low nitroxide/protein ratios (< 0.14 μmol/mg protein), without resolution of a static quenching phase. This suggests that sterols are segregated away from protein, probably in "clusters".

3. Paramagnetic quenching by stearate nitroxides increases abruptly between 35 and 50 °C. This discontinuous enhancement of quenching by temperature is reversible up to 41 °C but irreversible at higher temperatures. The discontinuity is also diminished by lowering pH from 7.3 through 6.5 to 6.0. Quenching by androstane nitroxide increases linearly with temperature up to approx. 41 °C and then rises exponentially. We attribute the reversible quenching thermotropism detected by stearate derivatives to reversible, thermotropic unfolding and/or depolymerisation of membrane proteins. The irreversible phase, detected also by the sterol derivative can be attributed to non-reversible protein denaturation.

4. Paramagnetic quenching of membrane tryptophan fluorescence by stearate derivatives is minimal at approx. pH 7.1 (35 °C) and increases sharply at lower and higher pH values, suggesting that two categories of protein residues, titrating between pH 6 and 8, profoundly influence the association of fatty acyl chains and penetrating protein segments. Quenching by androstane nitroxide exhibits no significant variation between pH 6 and 8, consistent with other data indicating that erythrocyte membrane sterols are segregated from membrane proteins, probably in clusters.

5. Our new approach confirms previous suggestions of a boundary layer of lipid in close association with some proteins in erythrocyte membranes, as well as experiments indicating that the lipid status in this boundary layer depends on that state of membrane proteins. However, sterols appear to be largely excluded from this boundary domain. Our data further show that lipid-protein interactions in erythrocyte membranes can vary significantly with fluctuations of temperature and pH in the physiological range.

INTRODUCTION

The excited-state lifetime of fluorophores can be reduced by paramagnetie molecules, thereby quenching fluorescence [1, 2]; such paramagnetic fluorescence quenching requires interaction distances of only 4–6 Å [3]. In a previous paper we have described how the fluorescence of perylene, a hydrophobic fluorophore, incorporated into lecithin and lecithin-cholesterol (molar ratio 1 : 1) liposomes is quenched under different conditions by several nitroxide lipid analogues [4]. We found that the paramagnetic quenching technique can sensitively monitor the thermotropic properties of model membrane systems. In addition the method has proven useful in evaluating clustering phenomena in lipid bilayers.

It has been suggested that paramagnetic fluorescence quenching might provide a way to localize nitroxide derivatives that are widely used as spin label probes for biomembranes, with respect to tryptophan, an intrinsic protein fluorophore [5]. We have previously documented the feasibility of this approach by documenting close associations between the paramagnetic residues of some stearic acid nitroxides and fluorophores in both serum albumin and erythrocyte ghost proteins [6, 7]. We have now applied paramagnetic fluorescence quenching to a more detailed study of lipid-protein relationships in erythrocyte membranes and have used sterol and stearic acid nitroxides, to measure suppression of native protein fluorescence as a function of quencher concentration, pH and temperature.

EXPERIMENTAL

Materials

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-[15 α-androstan-3,2′-oxazolidin]-3′-yloxyl (androstane nitroxide) were purchased from Syva (Palo Alto, California) and dissolved in methanol (Fisher certified) at 10^{-2} M. Other chemicals were of highest purity available commercially.

Human erythrocyte ghosts were prepared as in ref. 8 and, for quenching experiments, were diluted to 35 μg protein/ml in 50 mM phosphate, 1 mM NaN_3, pH 7.3; fluorometric protein determinations were performed as in ref. 9.

Measurements of paramagnetic fluorescence quenching

For quenching measurements 10^{-2} M methanolic solutions of nitroxides (0–40 μl; Hamilton microsyringes) were added to 2 ml of erythrocyte suspension,

mixed thoroughly and then transferred to fluorimeter cuvettes (Spectrasil). Methanol alone was added to control samples. For prolonged measurements all samples were remixed before each fluorescence measurement.

Quenching of protein fluorescence, Q, caused by nitroxide lipid analogues is expressed by the Stern-Volmer term $Q = (I_0/I) - 1$ [10], where I_0 is the fluorescence intensity without quencher and I is the emission intensity in the presence of quencher. Protein fluorescence emission was measured at 338 nm with excitation at 285 nm (slit width 7–10 nm) using a Perkin-Elmer MPF-3 spectrofluorimeter equipped with a cell compartment whose temperature was controlled to ± 0.05 °C using a Lauda K4R circulator. The fluorescence excitation and emission spectra of ghosts with and without quencher were identical in shape and band position. No inner filter effects were detected at the bulk nitroxide concentrations employed ($1 \cdot 10^{-5} - 2 \cdot 10^{-4}$ M). Titration of protein fluorescence with nitroxide was carried out at 25 °C; measurements were taken 30 min after changing nitroxide concentration. Corrections for turbidity at high androstane nitroxide concentrations were made when necessary. The instrumental accuracy was ± 1 %.

The temperature dependence of fluorescence quenching was evaluated in samples equilibrated with $5 \cdot 10^{-5}$ M nitroxide for 30 min at each temperature, starting at 15 °C and proceeding at 2–5 °C intervals. In reversibility experiments, samples lacking nitroxide were incubated at stated temperatures for 60 min. After re-equilibration at 25 °C (60 min), methanolic solutions of nitroxide or methanol, respectively, were added to samples and controls and quenching determined 60 min later. Q values of incubated samples were compared to those of identically treated reference samples kept continuously at 25 °C.

For pH experiments ghosts were pre-equilibrated for 15 h at 4 °C in 50 mM phosphate, 1 mM NaN_3, pH 6–8. After incubation at 35 °C (60 min) $5 \cdot 10^{-5}$ M nitroxide was added and Q determined 60 min later at 35 °C.

RESULTS

Fig. 1 illustrates the paramagnetic quenching of tryptophan fluorescence in erythrocyte membranes as a function of quencher concentration for the three nitroxide lipid analogues used in this study. At all concentrations tested, the sequence of quenching efficiency is 5-nitroxide stearate > 16-nitroxide stearate > androstane nitroxide. 16-Nitroxide stearate quenches less efficiently than 5-nitroxide stearate but both stearic acid analogues exhibit similar titration curves. Up to 0.28 μmol nitroxide stearate per mg membrane protein the Stern-Volmer plots show a positive deviation from linearity but, above 0.28 μmol/mg, the relationship between quenching and nitroxide concentration becomes linear; moreover, the linear segments extrapolate to the coordinate origin. In contrast, quenching by androstane nitroxide, which is as efficient as that by 16-nitroxide stearate up to 0.14 μmol/mg protein, saturates at slightly higher levels. No paramagnetic quenching is observed with aqueous solutions of tryptophan corresponding to the tryptophan content of the membrane proteins. The tryptophan fluorescence of L-leucyl-L-tryptophyl-L-methionyl-L-arginyl-L-phenylalanyl-L-alanine is also not quenched in solution.

Fig. 2 illustrates the temperature dependence of quenching by 5-nitroxide stearate, 16-nitroxide stearate and androstane nitroxide ($5 \cdot 10^{-5}$ M). Fluorescence

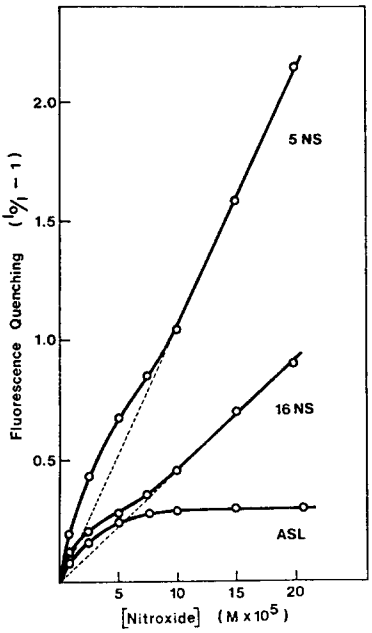

Fig. 1. Paramagnetic quenching of protein fluorescence in human erythrocyte membranes caused by increasing concentrations of stearic acid and sterol nitroxides at pH 7.3, 25 °C. Protein concentration, 35 μg/ml.

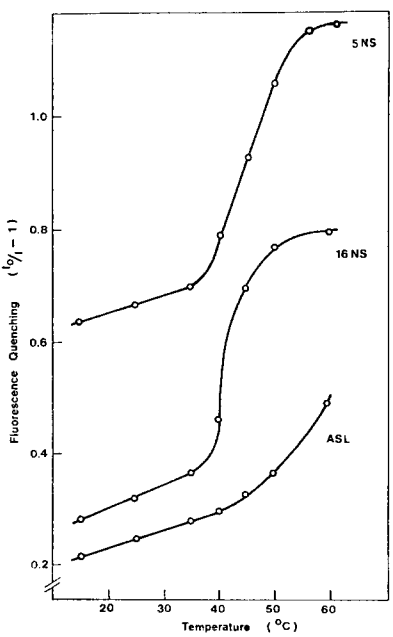

Fig. 2. Temperature dependence of paramagnetic quenching with 5-nitroxide stearate, 16-nitroxide stearate and androstane nitroxide. Erythrocyte membranes in 50 mM phosphate, 1 mM NaN_3, pH 7.3, at 35 μg protein/ml.

suppression by androstane nitroxide increases linearly up to approx. 42 °C and then increases exponentially. Quenching with 5-nitroxide stearate and 16-nitroxide stearate rises linearly between 15 and 35 °C, increases abruptly between 35 and 55 °C and reaches a new stable level at about 60 °C.

The temperature-induced quenching enhancement is not reversible in the presence of nitroxide. To distinguish between a thermotropism induced by or facilitated by the nitroxides and possible intrinsic thermotropism, we incubated erythrocyte membranes without quencher for 60 min at various temperatures above 25 °C, re-equilibrated at 25 °C, added the nitroxide and compared the fluorescence quenching with that in samples treated identically, except that they were kept at 25 °C throughout. Fig. 3 depicts the results obtained with 5-nitroxide stearate for the temperature range 40–45 °C. Samples incubated at temperatures up to 41 °C and re-equilibrated at 25 °C showed the same degree of quenching as samples maintained at 25 °C. However, samples preincubated at > 41 °C showed enhanced quenching also after re-equilibration at 25 °C. Similar results were obtained with 16-nitroxide stearate and androstane nitroxide. This indicates that the thermotropic effects responsible for the shown in Fig. 2 are reversible up to approx. 41 °C.

The H^+ concentration in the bulk phase strongly affects the temperature dependence of fluorescence quenching by 5-nitroxide stearate ($5 \cdot 10^{-5}$ M). At 25–35 °C, quenching at pH 6.0 is higher than at pH 7.3, whereas at > 45 °C quenching at pH 6.0 is lower than at neutrality. The results at pH 6.5 are intermediate. Approx. 40 °C fluorescence quenching is pH-insensitive.

As shown in Fig. 4, quenching of membrane tryptophan fluorescence by nitroxide stearates depends sensitively on pH. Quenching is minimal between pH 7.1 and 7.3 and increases sharply at lower and higher pH, indicating involvement of at

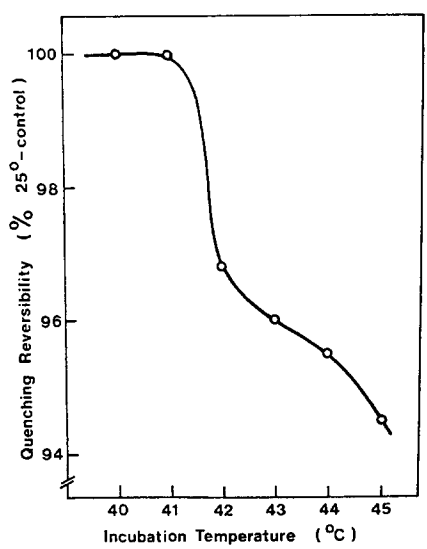

Fig. 3. Reversibility of fluorescence quenching between 40 and 45 °C. Samples lacking nitroxide were incubated for 60 min at stated temperatures and re-equilibrated at 25 °C before addition of $5 \cdot 10^{-5}$ M 5-nitroxide stearate. Quenching values are compared to those of non-incubated samples ($Q_{25\,°C} = 100\,\%$).

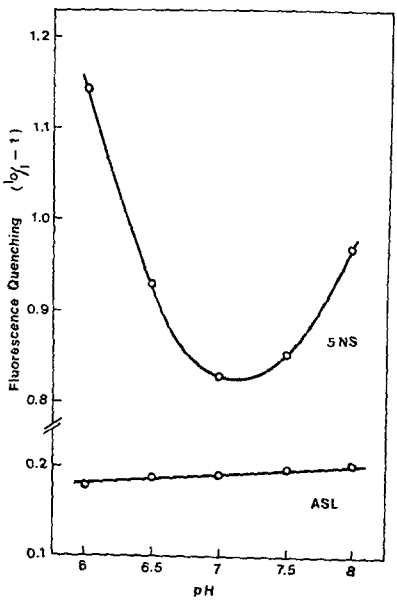

Fig. 4. pH dependence of fluorescence quenching with 5 nitroxide stearate and androstane nitroxide ($5 \cdot 10^{-5}$ M) at 35 °C. See text for details.

least two groups titrating between pH 6 and 8. In contrast, quenching by androstane nitroxide is essentially independent of H^+ concentration in this pH range.

DISCUSSION

Green et al. [3] have shown that fluorescence quenching by nitroxide takes place either through vibrational coupling between the excited states of donor and acceptor molecules or by enhanced singlet-triplet intersystem crossing. Since both mechanisms require the approximation of quencher and fluorophore to within about 5 Å, paramagnetic fluorescence quenching can occur either through collision or through formation of a ground-state complex between donor and acceptor molecule. According to the Stern-Volmer equation [10] diffusion-controlled collisional quenching is characterized by a linear relationship between quenching and quencher concentration. On the other hand, static quenching yields enhanced quenching efficiency, due to prolonged contact of quencher and fluorophore, and the corresponding Stern-Volmer plots therefore show a positive deviation from linearity [11].

The behaviour of 5-nitroxide stearate, 16-nitroxide stearate and androstane nitroxide in biomembranes and model systems has been intensively studied [12, 13]. These paramagnetic lipid analogues partition into membranes, with their polar headgroups anchored at water interfaces and the nitroxide-bearing hydrocarbon moieties extending to different depths into the hydrophobic domains of the membranes. The quenching effects reported here can thus be attributed to close interactions between the quenchers and the fluorophores on peptide segments penetrating into (through) the membrane, in contact with membrane lipids. Since the lateral mobility of nitroxide lipid analogues in lipid bilayers is high, one might expect only

diffusion-controlled, collisional quenching of protein fluorescence. This is not the case for 5-nitroxide stearate and 16-nitroxide stearate (Fig. 1), which, at low levels exhibit appreciable static quenching. This indicates that the two stearic acid analogues are bound to membrane protein, presumably through apolar interactions in a boundary layer [6]. Formation of nitroxide-tryptophan complexes is unlikely, since no quenching was observed with solutions of tryptophan or a tryptophyl hexapeptide. Moreover, as noted, the fluorescence excitation and emission spectra of ghosts were identical in band position and shape with and without nitroxide. This excludes the possibility of apparent quenching due to spectral shifts secondary to formation of ground-state complexes.

At quencher binding > 0.28 μmol/mg protein (10^{-4} M) a linear Stern-Volmer relation is observed, suggesting that the nitroxide stearates also partition into fluid membrane domains. It is also possible that high concentrations of nitroxides lead to a fluidization of the membrane. That this might occur is suggested by the fact that the linear segments begin at a nitroxide/membrane ratio which induces lysis of intact cells [14].

Paramagnetic fluorescence quenching measures the accessibility of protein tryptophan and possibly tyrosine [6] to nitroxide. Tyrosine residues may be involved because of the extensive transfer of electronic excitation energy from tyrosines to tryptophan in globular proteins as well as erythrocyte membrane proteins [15]. The higher quenching efficiency of 5-nitroxide stearate compared to 16-nitroxide stearate can thus be interpreted to indicate that the membrane fluorophores are concentrated, at a depth corresponding to C2 of phospholipid acyl chains. We cannot exclude, however, that this difference is due to a greater mobility of 16-nitroxide stearate, reducing contact duration of quencher and fluorophore.

Androstane nitroxide should introduce its nitroxide group at a depth equivalent to C8; its low quenching efficiency reflects either a deficiency of tryptophan in this region or a distribution of this sterol away from protein, possibly into cholesterol clusters. We tend to favour the second possibility because the saturation characteristic of the androstane nitroxide titration curve indicates a restricted volume and because a clustered distribution of androstane nitroxide has already been documented for liposomes [4, 16]. Further support for this argument comes from electron microscopic demonstration of clustering by cholesterol osmate esters [17]. Finally, the well known, ready exchangeability of sterols in erythrocyte membranes implies a low degree of protein-sterol interaction.

At neutral pH, quenching of membrane protein fluorescence by 5-nitroxide stearate and 16-nitroxide stearate increases abruptly with temperatures above 35 °C, indicating increased exposure of protein fluorophores to these paramagnetic lipid analogues. That this effect is not one induced by the action of 5-nitroxide stearate or 16-nitroxide stearate at temperatures above 35 °C, is proven by experiments in which the membranes were equilibrated at high temperatures in the absence of the nitroxides and the fluorescence measurements done at 25 °C. The latter experiments also show that the observed thermotropism is reversible up to 41 °C.

We suspect that more than one process underlies the abrupt but reversible quenching enhancement between 35 and 41 °C, as well as the irreversible change above 41 °C. Thus the quenching increment, at least between 35 and 41 °C might reflect reversible melting out of "boundary layer" phospholipid. This suggestion is

consistent with our laser-Raman observations [18] documenting a cooperative lipid state transition in erythrocyte membranes at approx. 19 °C. This transition is too highly cooperative to represent either boundary lipid or cholesterol-rich domains, and occurs at too low a temperature for membrane lipids such as sphingomyelin, which, in cholesterol-poor domains, would undergo the state change near 40 °C. Moreover, our data show that quenching is extremely pH sensitive at all temperatures and that the discontinuity between 35 and 45 °C is virtually abolished at pH 6. Since the pK_a values for the principal membrane phospholipids lie far from pH 7, we must consider primarily protein responses.

It is possible that monomeric proteins penetrating into the membrane can "unfold" reversibly up to 41 °C, exposing buried fluorophores to lipid. This process could become irreversible at higher temperatures, in analogy to the reversible and irreversible denaturation stages of soluble proteins. It is also possible that some penetrating membrane proteins are polymeric at $T < 35$ °C, that the contact sites between subunits bear tryptophan (or tyrosine) residues and that these residues become exposed upon depolymerization (irreversibly above 41 °C). This proposal is consistent with ESR data using 5-nitroxide stearate, showing a marked increase in the mobility of ghost lipids shortly after lowering pH from 7.4 to 6.2 [19].

The fact that androstane nitroxide-induced quenching increases discontinuously only at temperatures where thermal effects become irreversible fits with the suggestion that much of the sterol in erythrocyte ghosts is in a clustered state. Since clustering dissipates at higher temperatures, one can anticipate higher quenching under these conditions, particularly if the protein is thermally unfolded and/or depolymerized.

The pH dependence of paramagnetic fluorescence quenching with nitroxide stearates demonstrates that the interaction of membrane proteins and lipids depends rather subtly on the H^+ concentration in the bulk phase and that the most "compact" protein configuration obtains in the physiological pH range. The biphasic response indicates that at least two categories of ionizable residues, possibly histidines, are involved. The fact that quenching by androstane nitroxide is largely insensitive to pH is consistent with evidence presented above and elsewhere [4, 16–18] suggesting sterol clustering in erythrocyte membranes.

ESR measurements on erythrocyte membranes using high concentrations of 5-nitroxide stearate show that pH reduction decreases constraints on membrane lipid mobility that operate at neutral pH [19]. Such enhanced mobility would increase the frequency of collisions between fluorophore and nitroxide, thereby increasing paramagnetic fluorescence quenching. Electron microscopic studies demonstrate increased lateral mobility of intramembraneous particles at pH 5 secondary to extraction of "spectrin" at low pH's range [20, 21]. However, the pH effects detected by fluorescence quenching (Fig. 4) may also reflect the exposure of previously buried fluorophores.

Our fluorescence quenching measurements confirm other experimentation indicating that penetrating proteins are associated with boundary layers of membrane lipid. However, our results indicate that sterols are largely excluded from the boundary layer. Importantly, our experiments show that protein interactions in the boundary domain are not static but can respond to temperature and pH fluctuations in the physiological range.

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