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Influence of Cholesterol on the Rotation and Self-Association of Band 3 in the Human Erythrocyte Membrane†

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ABSTRACT: The cholesterol/phospholipid mole ratio (C/P) in the human erythrocyte membrane was varied by incubating cells with liposomes. The rotational mobility of band 3 proteins was measured in these membranes by observing flash-induced transient dichroism of the triplet probe eosin maleimide. Measurements were performed with membranes in which associations of band 3 with cytoskeletal proteins were removed by mild proteolysis with trypsin. It was found that decreasing C/P resulted in a more rapid decay of the flash-induced anisotropy. The anisotropy decay curves were analyzed by curve-fitting procedures, which indicated the existence of different sized small aggregates of band 3. The changes in the decay curves with varying C/P can be explained by an effect of cholesterol on the size distribution of these aggregates. The experiments suggest a possible role of cholesterol in regulating associations between integral membrane proteins.

The effect of cholesterol on the molecular motions of the hydrocarbon chains of lipids in bilayers and cell membranes is well documented (Oldfield & Chapman, 1972; Vanderkooi et al., 1974; Cooper et al., 1978; Kawato et al., 1978). Above the gel to liquid-crystalline phase transition, lipid-soluble probes report a decrease in "fluidity" in the presence of cholesterol. From time-resolved fluorescence depolarization measurements with the probe diphenylhexatriene, Kawato et al. (1978) deduced that the effect of cholesterol (above the phase transition) is principally to change the amplitude of the chain motions rather than their rate. Exactly how such changes in chain motions might affect the mobility of membrane proteins is by no means clear. However, it was rather surprising that in a previous study, no change in band 3 rotational mobility in the human erythrocyte membrane could be detected when the cholesterol/phospholipid mole ratio (C/P)[1] was varied from 0.34 to 1.66 (Nigg & Cherry, 1979). In these experiments, band 3 was selectively labeled with the triplet probe eosin maleimide. Rotation was measured by observing flash-induced transient dichroism of the eosin probe. Other measurements of band 3 rotational mobility have been made by Austin et al. (1979) and Johnson & Garland (1981).

It is well established that band 3 can be selectively cleaved with different proteases (Steck et al., 1978). Mild proteolysis of erythrocyte ghosts with trypsin releases a 41 000-dalton cytoplasmic fragment of band 3. It has been shown that there is a concomitant enhancement of the rotational mobility of the remaining 55 000-dalton membrane-associated fragment (Nigg & Cherry, 1980). A detailed analysis of these and other experiments led to the conclusion that about 40% of band 3 molecules have a restricted mobility due to association of the cytoplasmic moiety with cytoskeletal proteins. This finding suggests a possible explanation of the earlier failure to detect changes in band 3 rotation with varying C/P. The rotation of the fraction of band 3 that associates with cytoskeletal proteins is probably determined by protein–protein interactions and hence is insensitive to changes in the composition of the lipid bilayer. Changes in the rotational mobility of the remaining band 3 with varying C/P may be difficult to detect, since resolution of the two fractions by multiexponential curve fitting of the anisotropy decay curves is not particularly accurate.

To test whether the above explanation is correct, we have investigated the effect of varying C/P on band 3 rotation in membranes treated with trypsin. Cleavage of the cytoplasmic moiety releases those band 3 molecules that are associated with cytoskeletal proteins (Nigg & Cherry, 1980). Hence, more of band 3 (strictly, the 55 000-dalton membrane-associated fragment of band 3) becomes potentially sensitive to changes in membrane fluidity, and the test for an effect due to varying C/P is correspondingly more sensitive.

Materials and Methods

Fresh human blood (O^+) was centrifuged, some of the plasma to be used for the lipid dispersions was stored at 4 °C, and the erythrocytes were washed and labeled with eosin maleimide as described by Nigg & Cherry (1979). As shown

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[1] Abbreviation: C/P, cholesterol/phospholipid mole ratio.

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previously, reaction of eosin maleimide with intact erythrocytes produces a selective labeling of band 3 proteins. Cholesterol–lecithin dispersions were prepared according to Cooper et al. (1978). Eosin-labeled erythrocytes were given a final wash with incubation medium [50 mM Na_2HPO_4–NaH_2PO_4, pH 7.4, 64 mM NaCl, 10 mM KCl, 10 mM glucose, 10 mM inosine, 0.6 mM adenine, 0.08 mg/mL penicillin G (Sigma, 1667 units/mg), and 0.2 mg/mL streptomycin sulfate (Serva, 745 EU/mg)]. Then 4 mL of packed erythrocytes was diluted to 21 mL with the same medium, and 21 mL of the lipid–plasma dispersion was added. The suspension was dialyzed with gentle shaking for 25 h at 37 °C against the incubation medium. After the incubation, the liposomes were removed by centrifugation and the erythrocytes washed 3 times in isotonic buffer. Control samples were incubated without lipid dispersions under the same conditions.

Ghosts were prepared as described by Dodge et al. (1963), and the subsequent proteolytic splitting of band 3 with 1 μg of trypsin/mL was performed as described by Nigg & Cherry (1980). Since trypsin treatment of ghosts causes fragmentation of membranes, flash photolysis measurements were performed with samples in high-viscosity media to eliminate any possible contribution of vesicle tumbling to the anisotropy decay curves (Nigg & Cherry, 1980). High-viscosity media were prepared by the addition of 10% (w/v) Kollidon 90 (BASF) to the membrane suspension. After being vigorously mixed, the samples were kept overnight at 4 °C to allow thorough dissolution of the polymer. Prior to the measurements, the samples were flushed with argon for 10 min to remove O_2 and preincubated for 30 min at the desired temperature.

Protein was determined according to Lowry et al. (1951), phospholipids (after dialysis against a phosphate-free buffer) according to Chen et al. (1956), and cholesterol with the enzymatic Merkotest.

The flash photolysis apparatus used to measure rotational motion was similar to that described in detail elsewhere (Cherry, 1978). However, exitation was by a Nd-YAG laser (JK Lasers, Ltd.), using the frequency-doubled emission at 532 nm. The pulse width was about 15 ns and the repetition rate 16 Hz. Transient absorbance changes, caused by ground-state depletion, were simultaneously recorded at 515 nm for light polarized parallel and perpendicular with respect to the polarization of the exciting flash. The signals were collected and averaged by a Datalab DL 102A signal averager. Typically 1024 signals were collected in an individual experiment. Data were analyzed by calculating the absorption anisotropy $r(t)$, given by

$$r(t) = \frac{A_\parallel(t) - A_\perp(t)}{A_\parallel(t) + 2A_\perp(t)} \quad (1)$$

where $A_\parallel(t)$ and $A_\perp(t)$ are the absorbance changes at time t after the flash for light polarized parallel and perpendicular, respectively, with respect to the polarization of the exciting flash. $r(t)$ is independent of the signal lifetime and depends only on rotational motion, provided the absorption transient exhibits a single-exponential decay. The experimental $r(t)$ curves were fitted to double-exponential decays by the use of an iterative nonlinear least-squares program. The goodness of fit was judged by the sum-of-squares residuals, defined as

$$s^2 = \sum_{i=1}^{N} \frac{[r_i(t) - r(t)]^2}{N - n} \quad (2)$$

where N is the number of data points and n the number of parameters, and by the autocorrelation function of the residuals (Grinwald & Steinberg, 1974).

For the case of a protein that decays only around the membrane normal, the theoretical anisotropy decay is given by the expression (Cherry, 1978; Kawato & Kinosita, 1981)

$$r(t) = \left(\frac{r_0}{A_1 + A_2 + A_3}\right) \times \left[A_1 \exp\left(\frac{-t}{\phi_\parallel}\right) + A_2 \exp\left(\frac{-4t}{\phi_\parallel}\right) + A_3\right] \quad (3)$$

where the relaxation time $\phi_\parallel$ is defined as the reciprocal of the diffusion coefficient for rotation about the membrane normal. r_0 is the experimental value of the anisotropy at $t = 0$, and A_1, A_2, and A_3 are constants that depend only on the orientation of the transition dipole moment of the absorption band used for measurement. If the probe has multiple binding sites with different orientations in the same protein, eq 3 remains valid but the coefficients A_1, A_2, and A_3 now correspond to averages of the coefficients for each individual orientation.

If the population of rotating proteins is heterogeneous, the anisotropy decay contains two additional exponential terms for each additional component. Since resolution of the decays into more than two exponentials is rarely feasible, the following equation is often used for data analysis:

$$r(t) = r_1 \exp\left(\frac{-t}{\phi_1}\right) + r_2 \exp\left(\frac{-t}{\phi_2}\right) + r_3 \quad (4)$$

It should be emphasized that ϕ_1 and ϕ_2 in this case are not exact relaxation times but may give a rough estimate for the faster and slower components that are present.

Results

Rotational diffusion of eosin maleimide labeled band 3 was measured in three types of preparation, namely, control membranes, cholesterol-depleted membranes, and cholesterol-enriched membranes. Figure 1 shows a typical set of anisotropy decay curves measured at 45 °C. Qualitatively, it is immediately clear that the anisotropy decays more rapidly as C/P decreases. For a more quantitative analysis, the data were fitted by eq 3, which is the exact equation for a homogeneous population of proteins rotating around the membrane normal (Figure 1A). As judged by the parameter s^2 and also the autocorrelation function, the fits are not particularly good. The data were also fitted by the more general eq 4 (Figure 1B). In this case, much better fits were obtained, as is to be expected from the greater flexibility arising from the inclusion of an additional adjustable parameter. The parameters obtained from the curve fitting are collected together in Table I.

The effect of cholesterol depletion was also measured at different temperatures (Figure 2). At all temperatures in the range 10–45 °C reduction of C/P resulted in a more rapid decay of the anisotropy.

Discussion

As can be seen in Figures 1 and 2, depletion of cholesterol affects the mobility of band 3 at all temperatures in the range 10–45 °C. However, the general trend of a markedly decreased mobility at lower temperatures occurs in both the control and cholesterol-depleted membranes. The temperature effect and its possible interpretation in terms of self-aggregation of band 3 has been discussed in detail elsewhere (Nigg & Cherry, 1979). For present purposes, we have confined a detailed analysis of data to the results at 45 °C. As discussed elsewhere (Nigg & Cherry, 1980), the interpretation of the

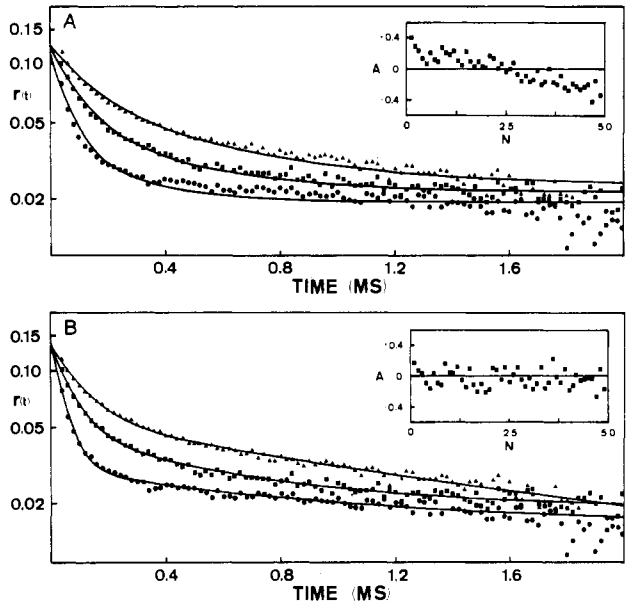

FIGURE 1: Anisotropy decay curves for trypsin-treated erythrocyte membranes at different C/P values. $r(t)$ was measured at 45 °C with membranes suspended in 5 mM sodium phosphate buffer, pH 7.4, containing 10% (w/v) Kollidon 90. (A) Data fitted by eq 3: (●) C/P = 0.48; (■) C/P = 0.87; (▲) C/P = 1.46. The autocorrelation function A of the residuals (inset) is shown for C/P = 0.87; a similar result was obtained for the other C/P ratios. The values of the sum of square residuals for the three curves were, for C/P = 0.48, s^2 = 0.00000536, for C/P = 0.87, s^2 = 0.00000413, and, for C/P = 1.46, s^2 = 0.00000673. (B) Data fitted by eq 4: (●) C/P = 0.48; (■) C/P = 0.87; (▲) C/P = 1.46. The autocorrelation function A of the residuals (inset) is shown for C/P = 0.87; a similar result was obtained for the other C/P ratios. The values of the sum of square residuals for the three curves were, for C/P = 0.48, s^2 = 0.00000230, for C/P = 0.87, s^2 = 0.00000254, and for C/P = 1.46, s^2 = 0.00000117.

Table I: Analysis of Anisotropy Decay Curves in Figure 1[a]

	cholesterol depleted (4), C/P = 0.48 ± 0.07	control (6), C/P = 0.87 ± 0.10	cholesterol enriched (3), C/P = 1.46 ± 0.02
	(A) Data Fitted by Equation 3		
$\phi_\parallel$ (μs)	225.9 ± 32.6	294.7 ± 44.7	432.9 ± 24.3
A_1 (%)	19.2 ± 2.0	25.8 ± 2.1	35.2 ± 2.5
A_2 (%)	63.0 ± 1.3	59.2 ± 3.9	44.7 ± 5.7
A_3 (%)	17.9 ± 0.8	15.1 ± 3.2	20.2 ± 7.7
	(B) Data Fitted by Equation 4		
ϕ_1 (μs)	45.1 ± 1.8	62.1 ± 8.2	96.7 ± 13.3
ϕ_2 (μs)	947.2 ± 373.8	471.1 ± 122.0	852.4 ± 92.5
r_1 (%)	77.8 ± 1.0	68.2 ± 4.3	56.5 ± 5.8
r_2 (%)	11.2 ± 2.1	19.6 ± 4.1	30.7 ± 2.0
r_3 (%)	11.0 ± 2.9	12.2 ± 2.6	12.8 ± 6.7

[a] The numbers correspond to the mean and standard deviations of independent measurements. Numbers in parentheses denote the number of measurements.

anisotropy decay curves may be somewhat simpler at this temperature than at lower temperatures.

We consider first the implications of the failure of eq 3 to provide a good fit to the experimental data. Essentially there are two possible explanations: either the model from which eq 3 is derived is incorrect or the population of rotating proteins is heterogeneous. The most obvious way in which the model could be incorrect is if, in addition to rotating around the membrane normal, band 3 proteins undergo a limited wobbling motion about the axes in the plane of the membrane. However, in the case of two other globular integral membrane proteins, bacteriorhodopsin (Cherry & Godfrey, 1981) and

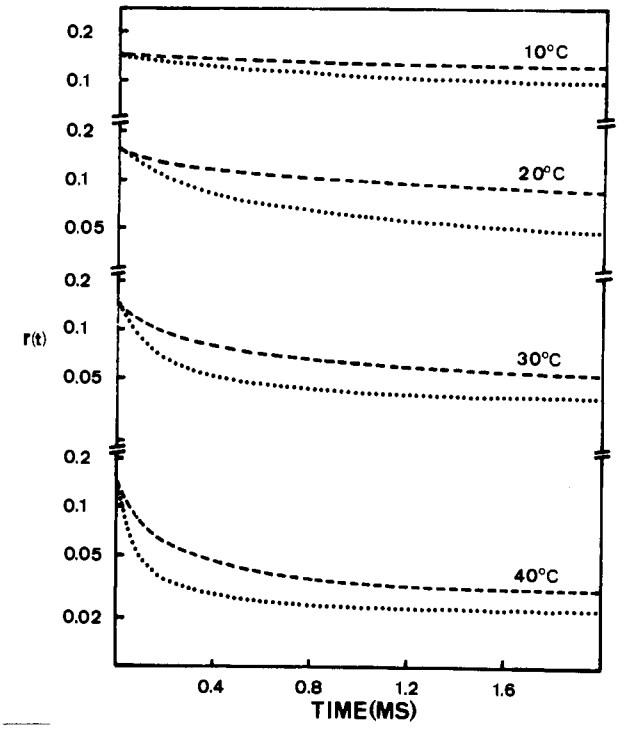

FIGURE 2: Effect of cholesterol depletion on anisotropy decay curves at different temperatures. Membranes were suspended in 5 mM sodium phosphate buffer, pH 7.4, containing 10% (w/v) Kollidon 90. (---) C/P = 0.87; (···) C/P = 0.48. For clarity, the data points are omitted and only the fitted curves (using eq 4) are shown. The signal to noise level was similar to the data shown in Figure 1.

cytochrome oxidase (Kawato et al., 1981), it appears that such a wobbling motion does not occur to any appreciable extent. Probably the changes in the local membrane structure that would accompany a significant wobbling motion are energetically unfavorable. Thus we consider that the failure of eq 3 to fit the data is most probably due to heterogeneity, such as would occur if band 3 were to be present in different aggregation states. In this case, the parameter $\phi_\parallel$ can be regarded as an approximate measure of the average relaxation time. As is evident from Table I (A), $\phi_\parallel$ decreases with decreasing C/P. However, it does not follow that this is necessarily due to decreasing membrane viscosity. It could equally well reflect a change in the average size of small aggregates of band 3.

A much better fit to the data is obtained by the more general eq 4. The parameters obtained, listed in Table I (B), show that r_3 remains constant at about 12%, while the remaining parameters change systematically with C/P. The one exception is the anomalously high value of ϕ_2 at low C/P. However, because of the low value of r_2, the value of ϕ_2 obtained from the curve fitting is very sensitive to the precise value of r_3. Hence, there is a large uncertainity in ϕ_2 at low C/P, and no particular significance should be attached to the anomalous value. The general conclusion that can be drawn from Table I (B) is that the faster time constant ϕ_1 decreases with decreasing C/P, while the fraction (r_1) of the faster component increases.

It seems unlikely that these changes can be explained entirely by changes in membrane viscosity. A change in viscosity might be expected to principally affect the time constants ϕ_1 and ϕ_2, with little effect on the coefficients r_1 and r_2. Even allowing for the uncertainties of curve fitting, it is clear, just from visual inspection of the decay curves, that at the highest C/P there is a larger fraction of a slowly rotating component present compared with that at the lowest C/P. This would

indicate that band 3 becomes more aggregated as C/P increases. The idea that cholesterol can affect the aggregation state of membrane proteins is supported by two previous observations. Bacteriorhodopsin in reconstituted phospholipid vesicles becomes strongly aggregated at C/P ratios greater than 20 mol % (Cherry et al., 1980), while fusion of liposomes containing cholesterol with mitoplasts causes aggregation of integral proteins of the mitochondrial membrane (Höchli et al., 1980; Hackenbrock, 1981).

Dorst & Schubert (1979) have previously argued that different-sized small oligomers of band 3 may exist in the erythrocyte membrane. The temperature dependence of band 3 rotational mobility is also most easily explained by self-association (Nigg & Cherry, 1979). In the case of several other integral membrane proteins in reconstituted systems, evidence has been obtained that self-association occurs at a low lipid/protein ratio (i.e., comparable to that of the erythrocyte membrane) (Kawato et al., 1981, 1982; Cherry & Godfrey, 1981). Thus the existence of different-sized aggregates of band 3 in the erythrocyte membrane appears to be a strong possibility. These aggregates must be fairly small (at 45 °C) since the low value of r_3 implies that most or all of band 3 becomes rotationally randomized within 2 ms. For this to occur, the maximum diameter of the aggregates should be no more than about 6 times that of band 3 dimers. The present data are consistent with an effect of cholesterol on the size distribution of the aggregates. Decreasing the cholesterol content of the membrane favors disaggregation while increasing the cholesterol content favors aggregation. Whether there is also a change in rotational motion due to a change in membrane viscosity cannot be ascertained. Although the variation of ϕ_1 with C/P in Table I (B) might suggest a variation in viscosity, this could also be a consequence of the change in the size distribution of band 3 aggregates.

In a previous study, no dependence of band 3 rotation on C/P was observed (Nigg & Cherry, 1979). It should be emphasized that the distinguishing feature of the present experiments is that band 3 was cleaved with trypsin to remove interactions with cytoskeletal proteins. Thus the mobility of a much larger fraction of band 3 proteins becomes sensitive to the lipid composition. The effect of cholesterol observed in the present experiments would be difficult to detect in non-trypsin-treated membranes, where rotation of a substantial fraction of band 3 is restricted by cytoskeletal associations.

The fractional value of r_3 obtained in the present experiments (from the better fit obtained with eq 4) is of interest. The minimum value of r_3 is related to the orientation of the transition dipole moment of the eosin absorption band in the membrane (Cherry, 1978; Kawato & Kinosita 1981). If r_3 is higher than the minimum value, this indicates the presence of proteins that are rotationally immobile on the time scale of the experiment. In previous experiments with trypsin-treated membranes (Nigg & Cherry, 1980), it was found that the anisotropy decayed at 45 °C to a constant value of r_3 of 25%, compared with 12% in the present study. We have found that this lower value is a consequence of the 25-h incubation of erythrocytes at 37 °C, since it occurred independently of whether or not liposomes were present in the incubation medium. In the absence of incubation, we found $r_3 = 22.9 \pm 2.05\%$ in the trypsin-treated membranes, in agreement with the previous study. A possible explanation for this effect is that incubation changes protein–protein interactions that immobilize a small fraction of band 3. Alternatively, though less probably, incubation could lead to a change in the orientation of the eosin chromophore.

In conclusion, the present study indicates that cholesterol can affect the self-association of integral membrane proteins. The effects on band 3 are more subtle than the gross lipid–protein segregations previously induced by cholesterol in bacteriorhodopsin–lipid vesicles (Cherry et al., 1980) and in mitoplasts (Höchli et al., 1980). Taken together, these investigations suggest a possible role for cholesterol in regulating protein–protein associations in membranes. In this context, the proposal of Zagyanski & Jard (1981) that cholesterol is involved in ligand-induced receptor microaggregation and endocytosis is of particular relevance. Their suggestion that cholesterol may be entrapped within protein aggregates gains some support in the case of band 3 from monolayer studies, which indicate preferential association of these components (Klappauf & Schubert, 1977). Alternatively, cholesterol could influence association of integral proteins through its well-known effect on membrane fluidity. In either case, the possiblity that cholesterol influences not only dynamic properties but also structural associations in the membrane should be seriously considered.

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