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ROTATIONAL DIFFUSION OF BAND 3 PROTEINS IN MEMBRANES FROM En(a—) AND NEURAMINIDASE-TREATED NORMAL HUMAN ERYTHROCYTES

ERICH A. NIGG [a,*], CARL G. GAHMBERG [b] and RICHARD J. CHERRY [a]

[a] *Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, CH-8092 Zürich (Switzerland) and* [b] *Department of Bacteriology and Immunology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29 (Finland)*

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Summary

Recent experiments have demonstrated an association between band 3 and glycophorin A in the human erythrocyte membrane (Nigg, E.A., Bron, C., Girardet, M. and Cherry, R.J. (1980) Biochemistry 19, 1887—1893). Here, the influence of sialoglycoproteins on the rotational diffusion of band 3 in the human erythrocyte membrane was investigated by studying membranes from En(a—) and neuraminidase-treated erythrocytes. Rotational diffusion was measured by observing flash-induced transient dichroism of eosin-labeled band 3. Although erythrocytes of the rare phenotype En(a—) lack the major sialoglycoprotein, glycophorin A, no significant difference in band 3 rotation at pH 7.4 and 37°C could be detected between En(a—) and normal erythrocyte membranes. Band 3 rotation at pH 7.4 was also insensitive to the enzymatic removal of sialic acid from the surface of normal erythrocytes. Moreover, the existence of an essentially similar temperature-dependent equilibrium between band 3 proteins with different mobilities was observed in normal, En(a—) and neuraminidase-treated erythrocytes. From these results it is concluded that glycophorin A contributes less than 15% to the cross-sectional diameter of the band 3-glycophorin A complex in the plane of the normal membrane. The rotation of the complex at pH 7.4 is not significantly influenced by either steric or electrostatic interactions involving the oligosaccharide moiety of glycophorin A.

* Present address: Department of Biology, University of California at San Diego, La Jolla, CA 92093, U.S.A.

Introduction

Band 3 and glycophorin A are the two quantitatively dominant membrane-spanning proteins of the human erythrocyte membrane. Band 3 proteins exist as dimers (or higher oligomers) in the membrane [1—3] and are responsible for anion transport (for review see Ref. 4). The physiological function of the major sialoglycoprotein of the membrane, glycophorin A, is still unclear. It is known that glycophorin A carries MN blood group activities and receptors for plant lectins and viruses (for review see Ref. 5). Furthermore, by carrying large amounts of *N*-acetylneuraminic acid (sialic acid), this protein contributes most of the net negative charge of the erythrocyte surface.

Recently, direct physical evidence for the existence of a non-covalent association between band 3 and glycophorin A in the erythrocyte membrane was obtained from protein diffusion measurements [6]. The rotational diffusion of band 3 proteins in the membrane was measured by observing flash-induced transient dichroism of the triplet probe, eosin maleimide [7]. The association of band 3 with glycophorin A was demonstrated by measuring band 3 rotation in the presence and absence of specific antiglycophorin A antibodies [6].

In the present studies, the role of glycophorin A in determining the mobility of the band 3-glycophorin A complex in the erythrocyte membrane was investigated. We took advantage of the existence of the rare naturally occurring human erythrocyte variant, the homozygous En(a—) erythrocyte [8,9], which completely lacks the major sialoglycoprotein, glycophorin A [10—12]. En(a—) erythrocytes therefore provide a particularly interesting system to investigate the influence of glycophorin A on the rotational diffusion of the band 3-glycophorin A complex in the human erythrocyte membrane. The possible effect of the negatively charged sialic acid residues of glycophorin A was further investigated by studying membranes from normal erythrocytes treated with neuraminidase.

Materials and Methods

Labeling of erythrocytes and ghost preparation

Normal human blood was obtained from the Swiss Red Cross Blood Transfusion Service. Blood from the En(a—) variant was obtained from the Finnish Red Cross Blood Transfusion Service, Helsinki. Erythrocytes were washed and labeled for 30 min at room temperature with the triplet probe, eosin 5-maleimide (Molecular Probes), as described previously [7]. Ghosts were prepared from labeled cells by hypotonic lysis in 5 mM NaH_2PO_4/Na_2HPO_4, pH 7.4.

Neuraminidase treatments

Eosin-labeled erythrocytes were washed three times in 10 mM Hepes, 5 mM $CaCl_2$, 145 mM NaCl, pH 7.2. For enzyme treatments packed cells were diluted with 1 vol. of the same buffer with or without neuraminidase ((E.C. 3.2.1.18) from *Vibrio comma* (cholerae), Behringwerke) at a final concentration of 0.1 I.U./ml. After an incubation for 90 min at 37°C, the cells were washed twice in ice-cold 5 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.4. Following

the preparation of ghost membranes, the samples were stored on ice for not more than 2 h prior to flash photolysis measurements. The amount of sialic acid on ghost membranes was determined according to the method of Warren [13] and protein was measured by using the method of Lowry et al. [14].

Flash photolysis measurements and data analysis

The flash photolysis apparatus used in these experiments is described in detail elsewhere [15]. The application of the technique to measure the rotational diffusion of band 3 has also been reported [7,16]. Briefly, protein-bound eosin probes were excited at 540 nm by a linearly polarized laser pulse of duration 1—2 μs. Transient absorbance changes at time t after the flash arising from ground-state depletion were simultaneously measured at 520 nm for light polarized parallel ($A_{\parallel}(t)$) and perpendicular ($A_{\perp}(t)$) with respect to the polarization of the exciting flash. The transient dichroism measurements were analyzed by calculating the absorption anisotropy $r(t)$, defined by the expression:

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

All results reported here were obtained by averaging 32 signals with a Datalab DL 102A signal averager. Data analysis was accomplished by a Hewlett Packard HP 9825A desk-top computer interfaced to the signal averager. The computer was also used to fit the experimental $r(t)$ values by exponential decays using an iterative non-linear least-squares program.

Results and Discussion

Band 3 rotation in En(a—) membranes

Fig. 1 shows a representative set of data for the time dependence of the absorption anisotropy for eosin-labeled membranes derived from normal and from En(a—) cells at two different temperatures. No major difference can be detected between curves obtained from normal and from En(a—) membranes at 37°C (Fig. 1A). At 15°C, however, the anisotropy curve arising from En(a—) membranes is slightly flatter than the corresponding control curve (Fig. 1B).

To provide a quantitative comparison of band 3 rotation in En(a—) and in normal ghosts, the decay of the absorption anisotropy was analyzed by curve-fitting procedures as previously described [7]. It was assumed that the experimental $r(t)$ reflects the rotation of the whole band 3-containing particle and that rotation only occurs about an axis normal to the plane of the membrane. On this basis, it was previously deduced that both rapidly and slowly rotating species of band 3 coexist in the membrane [7]. The equilibrium between these forms was found to be temperature dependent, the slowly rotating species becoming increasingly dominant as the temperature was reduced (Ref. 7, see also Fig. 1). It was proposed that these results might be due to temperature-dependent protein-protein associations [7].

In the present studies, the experimental data were fitted by the double exponential equation:

$$r(t) = B_1\exp(-t/\alpha_1) + B_2\exp(-t/\alpha_2) + 0.25\, r_0 \quad (2)$$

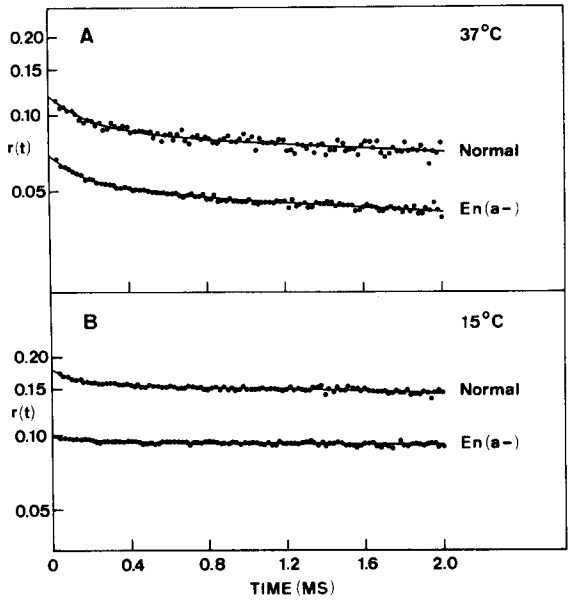

Fig. 1. Transient absorption anisotropy at 37°C (A) and 15°C (B) arising from eosin 5-maleimide-labeled band 3 in normal and En(a—) erythrocyte membranes. Flash photolysis experiments were carried out on ghosts suspended in 5 mM phosphate buffer, pH 7.4, as described in Materials and Methods. Prior to measurements, oxygen was displaced by a stream of argon [15] and the samples were equilibrated for 10 min at the indicated temperatures. The experimental points were fitted by using Eqn. 2 as described previously [7]. For illustrative purposes, the two curves in each panel have been artificially separated by vertical displacements of the lower curves by 20%.

where B_1, B_2, α_1 and α_2 are constants and the theoretically expected residual anisotropy is assumed to be 25% of the initial anisotropy, r_0 [7]. This resolution of the experimental decay of $r(t)$ into two exponential terms yields two time constants, α_1 and α_2, which may be regarded as order-of-magnitude relaxation times characterizing the rotation of rapidly and slowly rotating species of band 3. The coefficients, B_1 and B_2, are related to the fractional contribution of rapidly and slowly rotating populations.

Table I summarizes the results of this analysis when applied to data obtained from normal and from En(a—) membranes. At 37 and 25°C both the time constants and the coefficients characterizing the distribution of band 3 proteins between rapidly and slowly rotating species are not significantly different in En(a—) and in normal membranes. The only significant difference appears in the coefficients B_1 and B_2 at 15°C. At this temperature, the fractional contribution of the slow component is slightly enhanced in En(a—) membranes compared to normal erythrocyte membranes. The significance of this small difference is not clear at present. The interpretation evidently depends on the model adopted to explain the temperature dependence of band 3 rotation in normal erythrocyte membranes. If the phenomenon is based on a temperature-dependent self-aggregation of band 3 [7], the present finding would simply reflect a slightly favored self-aggregation of band 3 in En(a—) membranes at 15°C. Apart from this small effect, the results clearly demonstrate that band 3 rotation is not significantly affected by the absence of glycophorin A in En(a—)

TABLE I

ANALYSIS OF TRANSIENT ABSORPTION ANISOTROPY FROM EOSIN-5-MALEIMIDE LABELED BAND 3 IN NORMAL AND En(a—) ERYTHROCYTE MEMBRANES

Flash photolysis experiments were carried out on ghosts as described in the legend to Fig. 1. The experimental $r(t)$ curves were analyzed by using Eqn. 2 as described previously [7]. The results are expressed as means ± 1 S.D.; n is the number of experiments.

	n	α_1 (ms)	α_2 (ms)	B_1 (%)	B_2 (%)
15°C					
En(a—)	7	0.12 ± 0.03	41.5 ± 18.8	5.3 ± 0.5	69.7 ± 0.5
Normal	12	0.16 ± 0.06	20.7 ± 10.4	11.4 ± 3.0	63.6 ± 3.0
25°C					
En(a—)	7	0.20 ± 0.05	10.8 ± 2.3	14.7 ± 2.0	60.3 ± 2.0
Normal	5	0.15 ± 0.02	8.8 ± 2.0	15.3 ± 0.9	59.7 ± 0.9
37°C					
En(a—)	15	0.17 ± 0.02	4.9 ± 1.2	26.5 ± 3.5	48.5 ± 3.5
Normal	16	0.19 ± 0.05	5.2 ± 1.5	24.4 ± 1.8	50.6 ± 1.8

membranes.

This observation is clearly relevant to the structure of the band 3-glycophorin A complex which was previously demonstrated to exist in the normal membrane [6]. The relaxation time for rotation of a membrane protein about the membrane normal depends on the square of its cross-sectional diameter in the plane of the membrane, and hence is rather sensitive to this dimension [17]. At 37°C the time constants of the decay of the absorption anisotropy for En(a—) and normal erythrocyte membranes are the same within the experimental uncertainty, implying that glycophorin A contributes less than 15% to the hydrodynamic diameter of the complex. This corresponds to less than 9—12 Å if we take the overall diameter of the complex to be 60—80 Å [18].

This view is supported by results from freeze-fracture studies [19,20] which show that the shape and the distribution of intramembrane particles (which are believed to contain the major integral proteins) are unaltered in En(a—) cells when compared to normal erythrocytes. From these studies it was also concluded that glycophorin A is not a quantitatively important constituent of the intramembrane particles [19,20]. Moreover, a recent report by Lutz et al. [21] indicates that glycophorin A by itself may not give rise to intramembrane particles in erythrocyte membranes.

The results of the present studies are also consistent with the topographical disposition of the polypeptide chains of the two proteins. Glycophorin A has a sequence of about 23 apolar amino acid residues which is believed to span the bilayer [22], probably as an α-helix [23]. The arrangement of band 3, on the other hand, is much more complicated. Several proteolytic fragments of band 3 may be generated which are not released from the membrane and therefore must be tightly associated with the lipid bilayer (for review see Ref. 24). The most hydrophobic segment of band 3 is a 17 000 dalton fragment which contains about 154 amino acid residues [25,26]. This segment probably exhibits a complex folding and may be arranged in a globular structure within the membrane. The stoichiometry of the two proteins in the membrane is about 1 glycophorin A per band 3 monomer [27]. Assuming the stoichiometry

is reflected in the individual complex, glycophorin A would contribute less than 15% to the mass of the hydrophobic part of the complex. There is no difficulty in visualizing packing models in which the relatively small glycophorin A segments are incorporated into the complex without significantly increasing the overall cross-sectional diameter.

Band 3 rotation in membranes from neuraminidase-treated erythrocytes

The above arguments assume that rotational diffusion of the band 3-glycophorin A complex is determined by the viscous resistance of the lipid bilayer. It is also conceivable that either steric or electrostatic interactions in the oligosaccharide moieties of the proteins could have some effect on rotational motion. The similarity of rotation of band 3 in En(a—) and normal cell membranes, however, argues against steric effects due to glycophorin A oligosaccharides which contribute most of the total carbohydrate of the normal erythrocyte surface. Although there is an increase in glycosylation of band 3 in En(a—) cells [10,28], it is unlikely that this would be sufficient to compensate exactly effects due to the absence of glycophorin A. Evidence for the lack of electrostatic influence on rotational motion is further provided by studies on normal membranes treated with neuraminidase. Incubation of intact cells with this enzyme at a concentration of 0.1 I.U./ml for 90 min at 37°C resulted in the removal of approx. 90% (89.1 ± 5.6; mean ± S.D. of seven experiments) of the total sialic acid from the membrane surface. The removal of the negatively charged sialic acid would be expected to decrease electrostatic repulsion between individual band 3-glycophorin A complexes. This in turn could conceivably lead to enhanced self-aggregation. However, Table II shows that the rotational diffusion of band 3 is remarkably insensitive to the enzymatic removal of sialic acid from the cell surface. The previously reported temperature dependence of band 3 rotation [7] is also unaffected by prior neuraminidase-treatment of the membranes. This finding implies that electrostatic repulsion by negatively charged sialic acid residues is not a major determinant in controlling the aggregate size of the band 3-glycophorin A complexes.

TABLE II

ANALYSIS OF TRANSIENT ABSORPTION ANISOTROPY FROM EOSIN-5-MALEIMIDE LABELED BAND 3 IN UNTREATED AND NEURAMINIDASE-TREATED ERYTHROCYTE MEMBRANES

Flash photolysis experiments were carried out on ghosts as described in the legend to Fig. 1. The experimental $r(t)$ curves were analyzed by using Eqn. 2 as described previously [7]. The results are expressed as means ± 1 S.D.; n is the number of experiments.

	n	α_1 (ms)	α_2 (ms)	B_1 (%)	B_2 (%)
15°C					
Neuraminidase-treated	7	0.20 ± 0.05	15.6 ± 2.7	11.5 ± 1.4	63.5 ± 1.4
Untreated	5	0.15 ± 0.05	17.6 ± 2.3	10.7 ± 1.0	64.3 ± 1.0
25°C					
Neuraminidase-treated	6	0.18 ± 0.05	7.9 ± 1.0	17.3 ± 0.7	57.6 ± 0.7
Untreated	4	0.15 ± 0.07	8.4 ± 2.0	15.5 ± 0.8	59.5 ± 0.8
37°C					
Neuraminidase-treated	11	0.15 ± 0.02	5.2 ± 0.6	24.4 ± 2.2	50.6 ± 2.2
Untreated	9	0.18 ± 0.03	4.4 ± 0.8	25.0 ± 1.7	50.0 ± 1.7

In conclusion, we emphasize that the results of the present study do not contradict the previous finding that band 3 and glycophorin A form a complex in the erythrocyte membrane [6]. The lack of influence of glycophorin A on band 3 rotation reported here may readily be explained by a negligible contribution of glycophorin A to the cross-sectional diameter of the complex. This result is in complete accord with biochemical data on the sizes of the two proteins [23,24]. It is, however, noteworthy that glycophorin A does not significantly influence the mobility of the complex by steric and/or electrostatic interactions involving the large oligosaccharide moiety of this major sialoglycoprotein.

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