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Low-pH association of proteins with the membranes of intact red blood cells. II. Studies of the mechanism

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The low-pH interaction of proteins with erythrocyte membranes has been found to be correlated with pH-induced changes in the erythrocyte membrane. Using a 90° lightscattering method it was shown that red blood cell hemolysis was slow between pH 5.8 and 5 ($t_{1/2}$ above 1 h) but became fast at and below pH 4.7 ($t_{1/2}$ less than 20 min). At pH 4.7, the presence of glycophorin in the incubation medium inhibited the hemolysis of erythrocytes and this protective effect was found to be dependent on the glycophorin concentration. Electron microscope experiments showed the presence of membrane defects after 10 s incubation at pH 4.6 in the absence of glycophorin in the incubation medium. These defects could further develop into openings with average widths of 14 nm after 1.5 min incubation under the acidic conditions. Fluorescence and flow cytometry studies showed that at pH 4.7, but not at pH 7.4, glycophorin tightly associates with phosphatidylcholine liposomes, and that liposome associated glycophorin molecules are recognized by anti-glycophorin monoclonal antibodies.

Introduction

In the preceding paper [1] we reported that glycophorin as well as CD4-molecules became tightly associated with erythrocyte membranes after incubation at pH values below 5. Some aspects of the mechanisms involved in this low-pH interaction will be analyzed in this paper, with emphasis on the contribution of pH effects on the erythrocyte membranes to the association process.

A number of studies on the interaction of proteins with lipid model membranes have shown that low pH is an important factor. Fragments A and B of diphtheria toxin were shown to interact with liposomes with optimal conditions below pH 5.4, and no pH gradient across the liposomal membrane was required for this process [2]. Membrane penetration of diphtheria toxin in vivo is believed to be triggered by low pH, and it was shown that below pH 5 the interaction of this protein with liposomes is rapid and strong [3,4]. The insertion of colicins into liposomes and the channel-forming activity of these proteins was shown to require pH values below pH 5 [5,6], a requirement also observed for the channel-forming toxins. diphtheria [7–10] and tetanus toxins [11].

The molecular mechanisms of protein–membrane interaction, including translocation and penetration, are at present not completely understood. Emphasis has been placed on the changes that must occur in the protein before its interaction, but it is by no means certain that membrane transformation may not favor an association process [12]. This was shown to be the case for protein interaction with liposomes [13–15].

Using 90° lightscattering, electron microscopy, flow cytometry and fluorescence methods we show in this study that defects induced by low pH in the erythrocyte membrane may play a role in the low-pH protein–erythrocyte membrane interaction.

Materials and Methods

Chemicals. Egg phosphatidylcholine (PC), was from Sigma (St. Louis, MO), sodium cacodylate, glutaralde-

Abbreviations: a.u., arbitrary units; CD4, antigen on helper-inducer T lymphocytes, monocytes; FITC, fluorescein isothiocyanate; Gam-IgG-Pe, phycoerythrin-labeled goat antimouse immunoglobulin G; kDa, kilodalton(s); PBS(7.4), 5 mM sodium phosphate buffer, 145 mM NaCl (pH 7.4); PC, phosphatidylcholine; RBC, red blood cells.

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hyde and EMBED-812 were from Electron Microscopy Sciences (Ft. Washington, PA), propylene oxide was from Polysciences (Warrington, PA).

Liposome preparation. Liposomes with or without reconstituted glycophorin in the bilayers were prepared by the method of MacDonald and MacDonald [16]. For reconstitution of glycophorin into lipid bilayers, 1 mg egg phosphatidylcholine was dissolved in 1 ml of a methanol/chloroform mixture (1 : 2, v/v) and mixed with 15 μl of a glycophorin solution in distilled water (0.1 mg glycophorin). After drying under nitrogen, the protein-lipid film was swollen for 1 h in 1 ml 5 mM sodium phosphate buffer, 145 mM NaCl (pH 7.4) (PBS (7.4)) and liposomes were formed by gentle hand-shaking or gentle vortexing. Liposomes without glycophorin were prepared following the same procedure, the only difference being that the 15 μl of distilled water that was added to the PC suspension in methanol/chloroform contained no glycophorin.

Measurements of kinetics of erythrocyte lysis. The time dependence of red blood cell hemolysis induced by acidic pH was recorded using a 90° lightscattering method. Erythrocytes were isolated as described in the preceding paper [1]. 40 μl from a RBC suspension in PBS (7.4) (about $2 \cdot 10^7$ cells) were mixed at time zero with 2 ml of 0.02 N sodium acetate buffer, 145 mM NaCl, at different pH values, in the fluorescence cuvette. The decrease of 90° lightscattering due to cell lysis ($\lambda_{ex} = 720$ nm, $\lambda_{em} = 720$ nm) was monitored using a SLM-8000 spectrofluorometer. The 90° lightscattering measurements were performed in a thermostated cell holder ($T = 37$°C) with continuous mixing of the contents by a magnetic stirrer.

Low-pH incubation of glycophorin with erythrocytes and with liposomes. Glycophorin was incubated with erythrocytes and liposomes using the low-pH method as described in the preceding paper [1]. 1.5 ml of sodium acetate buffer (pH 4.6) plus 100 μl of glycophorin in PBS(7.4) and 30 μl of RBC suspension (about $1.4 \cdot 10^7$ cells) were mixed and incubated at 37°C for 1.5 min. After incubation, the RBC were concentrated by centrifugation (2 min at 3000 rpm in an Eppendorf centrifuge). The pellets were resuspended in PBS(7.4) and a second centrifugation was performed under the same conditions. In the case of liposomes, 50 μl of a liposome suspension (0.1 mg lipids) were added over 1.5 ml of the same sodium acetate buffer (pH 4.6) containing 30 μl of glycophorin solution in PBS(7.4) (25 μg glycophorin). After incubation for 1.5 min at 37°C, the liposomes were concentrated by centrifugation (10000 rpm for 4 min, in an Eppendorf centrifuge). The liposome pellet was resuspended in PBS(7.4) and washed twice in PBS(7.4) by centrifugations.

Protection of low-pH hemolysis by glycophorin. Erythrocytes were incubated with different concentrations of glycophorin employing the same conditions used for the low-pH incubation previously described. After washing, the resulting RBC pellets were suspended in 1.5 ml PBS(7.4) and hemolyzed by the addition of Triton X-100 (0.27%, v/v). The absorbance, A, of these Triton-treated samples was measured at the 420 nm Soret band of hemoglobin using an Hitachi U-2000 spectrophotometer. Two reference samples, corresponding to maximal and minimal hemolysis, were used. Maximal hemolysis was represented by a sample in which the erythrocytes were incubated at low pH in the absence of glycophorin; the RBC pellet of this sample contained the smallest amount of hemoglobin, i.e., the smallest absorbance, A_0. Minimal hemolysis was represented by a sample of erythrocytes that were incubated in PBS(7.4); this sample had the highest hemoglobin content of the RBC pellet, i.e., the largest Triton-treated pellet absorbance, A_{100}. Using A_0 and A_{100}, the percentage of protection from hemolysis by glycophorin at a given concentration was defined as:

$$\% \text{ protection} = [(A_g - A_0)/(A_{100} - A_0)] \times 100 \quad (1)$$

where A_g is the absorbance of the Triton X-100-treated pellet of RBC which were incubated with glycophorin.

Preparation of samples for electron microscopy. Human erythrocytes were incubated for different time intervals at pH 4.7 (0.02 N sodium acetate buffer, 145 mM NaCl) in the absence of proteins. Cells were fixed by the addition of 0.74 ml of ice-cold double-strength fixative at the following times after RBC addition to the pH 4.7 buffer: 10 s, 35 s, 65 s, 95 s. 'Double-strength' fixative consisted of the following: 6% glutaraldehyde, 0.2 M sodium cacodylate and 0.06% $CaCl_2$ titrated to pH 4.7 with HCl and was used so that after addition to (and subsequent dilution with) the incubation medium, final fixative concentrations would be the same as routinely used. Cells were fixed in suspension at 4°C for 1.5 h and pelleted. The pellet was washed at 4°C with an 0.3 M solution of sucrose in 0.1 M cacodylate, 0.03% $CaCl_2$, stained with osmium (2% osmium tetroxide, 0.1 M cacodylate, 0.15 M sucrose and 0.03% $CaCl_2$, for 1.5 h at 4°C), dehydrated through an ethanol gradient with two final 5 min incubations in 100% propylene oxide and embedded in EMBED-812. Thin sections were poststained with uranyl acetate and viewed using a Philips 420 electron microscope operated at 60 kV.

Control experiments for the experiments listed above were: (1) fixation of RBC using 'double-strength' fixative at pH 4.7 or at pH 7.4 without previous incubation of cells in acetate buffer at pH 4.7, and (2) fixation at pH 7.4 after 35 s incubation in acetate buffer at pH 4.7. Mixtures of larger volumes indicated that when 0.74 ml of 'double-strength' fixative (pH 7.4) were added to 0.74 ml of acetate buffer (pH 4.7), the final pH of the mixture was 7.1.

Fluorescence measurements of glycophorin–liposome interaction. Glycophorin was labeled with fluorescein isothiocyanate (FITC) as described in the preceding paper [1]. For kinetic measurements, at time zero, 40 μl of PC-liposome suspension was added (500 nM PC lipids, final concentration) to the fluorescence cuvette containing 2 ml of buffer and 20 μl of FITC-glycophorin solution (about 40 nM glycophorin, final concentration in the cuvette). Measurements were performed on a SLM-8000 spectrofluorometer in a thermostated sample holder at 37°C and with continuous mixing by a magnetic stirrer.

For polarization degree measurements, two populations of liposomes were used: (i) liposomes with FITC-glycophorin reconstituted into the bilayers by the method of MacDonald and MacDonald [16]; and (ii) liposomes that were reacted at low pH with FITC-glycophorin, as previously described. Both liposome suspensions were eluted through Sepharose 4B columns using PBS(7.4). 50 μl of liposome eluate were added to 2 ml of PBS(7.4) and the FITC polarization degrees were measured as described in Ref. 1.

Incubation of liposomes with antibodies. 0.1 mg liposomes were incubated with 6 μg antiglycophorin antibody 10F7 for 30 min at 22°C. The liposomes were concentrated by centrifugation (10000 rpm for 4 min in an Eppendorf centrifuge) and washed twice with cold PBS(7.4). The liposome pellet was then incubated with 10 μg of Gam-IgG-Pe in a total volume of 100 μl PBS(7.4) for 30 min at 22°C. Unbound fluorescent antibodies were removed by two washes with cold PBS(7.4).

Flow cytometry. Liposomes were analyzed by flow cytometry under the same conditions described in the preceding paper [1]. Analysis was restricted to a population of large liposomes which have sizes comparable to erythrocytes as indicated by the side-scatter/forward-scatter histograms in the flow cytometer.

Other procedures. All other experimental procedures and sources of chemicals are given in the preceding paper [1].

Results

Kinetics of hemolysis at low pH

In the low-pH interaction of membrane proteins with the erythrocyte membrane, a faint hemolysis was observed in the controls, i.e., erythrocytes incubated at pH 4.7 for 1.5 min in the absence of proteins in the reaction medium. No hemolysis could be seen when glycophorin or the CD4 extract was present in the reaction medium, conditions in which proteins become associated with the erythrocyte membrane [1]. The pH-induced hemolysis of human erythrocytes was investigated using a 90° light scattering method. The results of these experiments are shown in Fig. 1. After addition of RBC to PBS(7.4), no changes in the light scattering were observed (i.e., no hemolysis). (The small initial increase in the scattering may arise from a hypotonic swelling of the erythrocyte, since the erythrocyte stock solution, due to high erythrocyte concentration, may have a slightly higher osmolality than the 150 mM PBS(7.4) buffer.) Below pH 6, hemolysis started to occur, being a slow process between pH 5.8 and 5.0 and becoming fast between pH 5.0 and pH 4.0 (Fig. 1). From the experiments in Fig. 1 and other similar ones, the pH dependence of the half-time of hemolysis, $t_{1/2}$, was obtained (Fig. 2). A biphasic effect of low pH on erythrocyte hemolysis was observed, with a fast hemolysis between pH 3.6 and pH 4.7 and a slower one above pH 5 (Fig. 2). These data indicate that in a narrow pH range (between pH 5.0 and pH 4.7) important changes in the erythrocyte membrane occur.

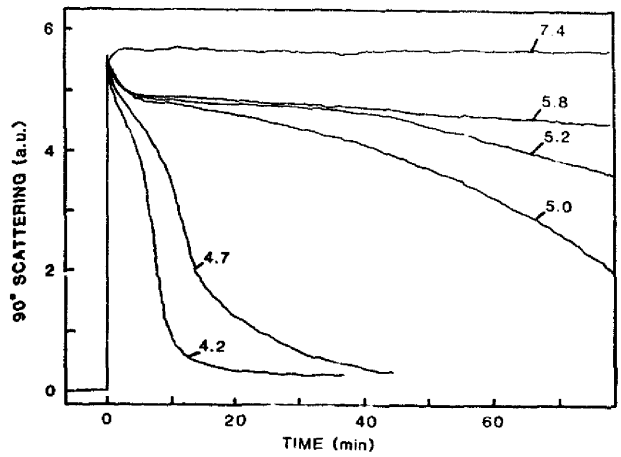


Fig. 1. Time dependence of pH-induced hemolysis measured by changes in 90° lightscattering. Erythrocytes were mixed at time zero with buffers of different pH values (pH indicated above each curve).

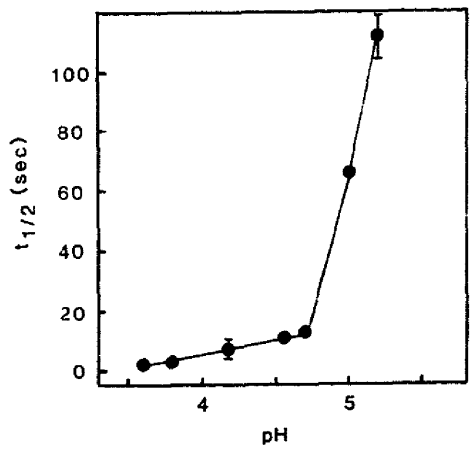


Fig. 2. Half-time of hemolysis as a function of pH. The half-time values, $t_{1/2}$, were measured from hemolysis curves obtained by the 90° lightscattering method and represent the time of 50% reduction in the initial 90° lightscatter intensity, see Fig. 1.

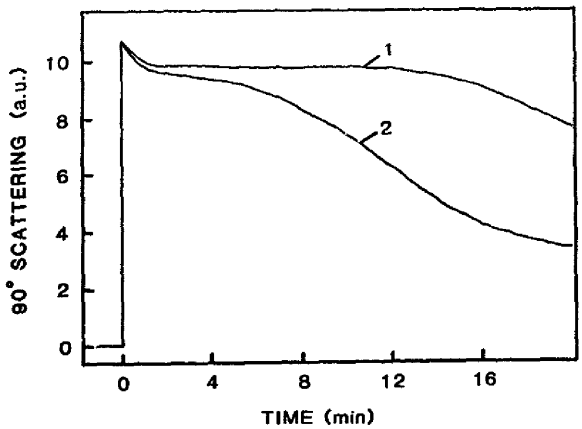


Fig. 3. Protection of low-pH induced hemolysis by glycophorin. Changes in the 90° lightscattering intensity of the erythrocyte suspension were monitored with glycophorin in the incubation media (curve 1) and in the absence of glycophorin (curve 2). In both cases, the pH of the incubation media was 4.7, $T = 37$ °C.

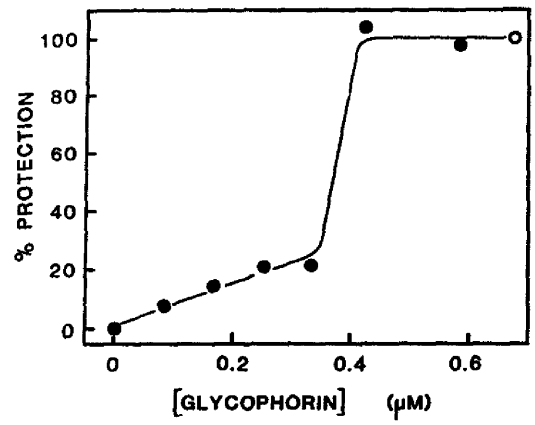


Fig. 4. Protection against low-pH hemolysis by glycophorin is dependent on glycophorin concentration. The percent protection against hemolysis was calculated for the hemoglobin content of erythrocyte pellets after the low-pH incubation using Eqn. 1. Erythrocytes incubated under the same conditions at pH 7.4 (in PBS(7.4)) were used for the determination of the value corresponding to 100% protection (○).

Protective effect of glycophorin against low-pH hemolysis

When glycophorin was present in the low-pH incubation medium used (pH 4.7), the erythrocytes were protected against hemolysis for up to 10 min (Fig. 3). For a short incubation time (1.5 min) the protective effect of glycophorin against the hemolysis of an erythrocyte population was studied at different glycophorin concentrations. As shown in Fig. 4, a sharp decrease in the percentage of protection occurred below 40 nM glycophorin per $1.5 \cdot 10^7$ erythrocytes. The low-pH induced hemolysis was also inhibited by dextran molecules of a mean molecular mass greater than 70 kDa, human or bovine serum albumin or 10 nm gold beads coated with immunoglobulin (data not shown).

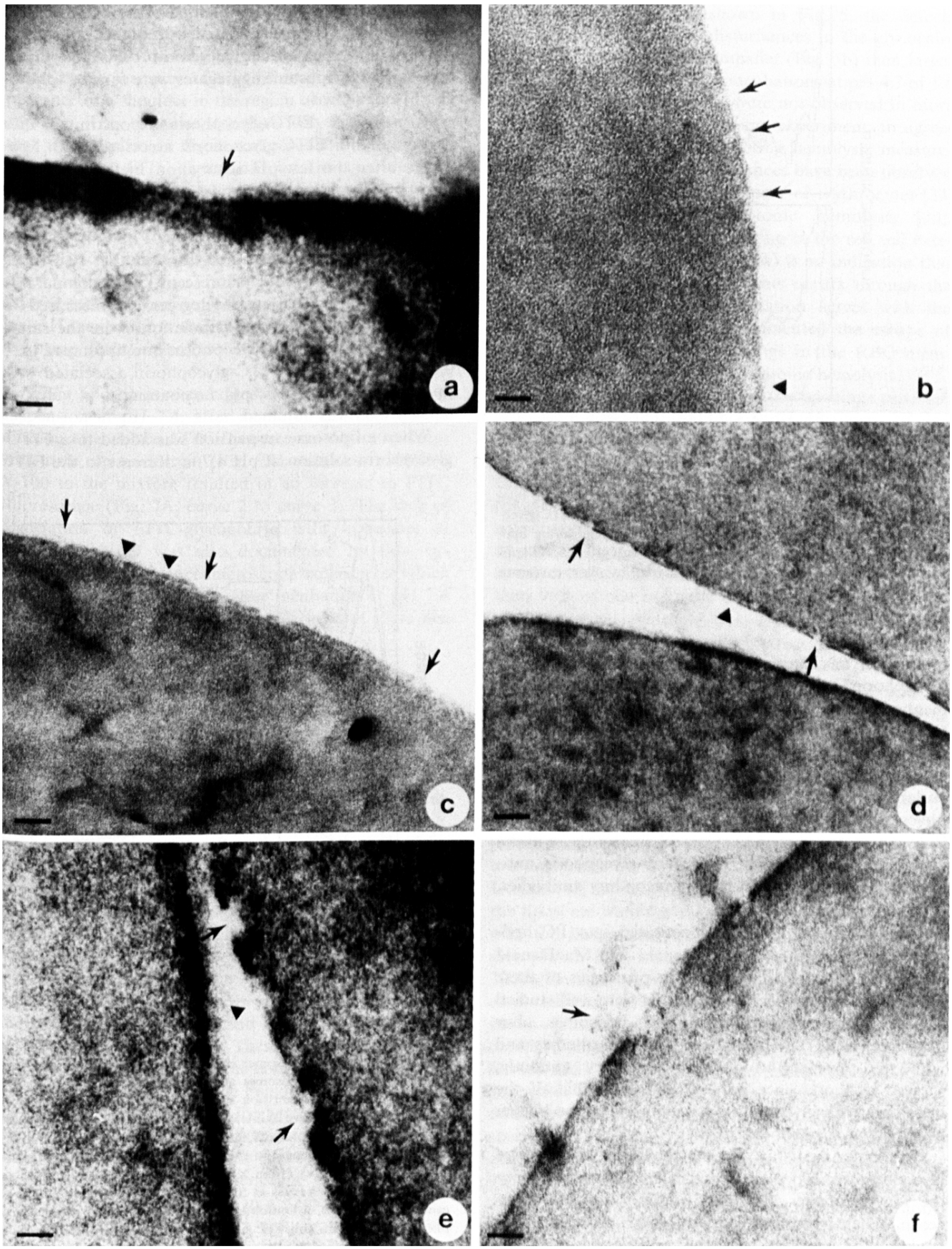
Ultrastructural changes induced by low pH in RBC membranes

Incubation of human red blood cells at pH 4.7 in the absence of glycophorin induced disturbances in the glycocalix which developed into slits after longer incubation times. For incubation times up to 35 s, thin sections showed patches of reduced density in the glycocalix, i.e., heavy metal staining was reduced, indicating fewer glycocalix components in that area (Fig. 5a, arrow). At other sites, the glycocalix appeared intact, but beneath it were more transparent areas of reduced density, which appeared to be chains of spheres with diameters of about 10 nm (Fig. 5b,c,d, arrowheads), possibly a stage preceding opening formation. At 35 s, occasional openings were observed (Fig. 5b, arrow) with widths of about 6.5 nm and depths of about 10.8 nm in the plane of the thin section. With incubation at pH 4.7 for longer times (up to 95 s), larger openings were observed (Fig. 5c,d,e,f). The average width of these defects was 14.1 nm and their average depth was 12 nm. For comparison, Fig. 5d and e show erythrocytes with apparently normal membranes beside RBC having low-pH induced openings. Occasionally, it seemed that RBC contents were being lost (Fig. 5f, arrow), which could correspond with the observed low level of hemolysis in the absence of proteins in the reaction medium (see Fig. 1). The defects developed, in time, from disturbances in the glycocalix to smaller openings (6.5 nm width) to larger openings (14.1 nm) at 1.5 min. No significant large openings (14 nm) were observed with low-pH incubation for up to 35 s, but at incubation times of 65 s and 95 s, large and small openings and all degrees of disturbances in the glycocalix were also observed. At no time did more than 10% of erythrocytes incubated at pH 4.7 in the absence of protein show any membrane openings.

Low-pH interaction of FITC-glycophorin with PC liposomes

To examine whether the erythrocyte membrane proteins are in themselves a prerequisite for the low-pH

Fig. 5. Electron micrographs of ultrastructural changes in erythrocyte membranes induced by low-pH. Human RBC incubations at pH 4.7 were quenched by the addition of fixative at different times. Shown in a is a sample which was quenched with fixative after 10 s (arrow points to a disturbance in the glycocalix); b, after 35 s (arrows point to 6.5 nm wide openings) c and d, at 65 s; and e and f, after 95 s. Arrows point to openings of 14.1 nm wide in e. Arrowheads in b, c and d point to transparent chains of spheres beneath an intact glycocalix. The arrowhead in e points to an opening partially covered by glycocalix. The arrow in f points out electron-dense materials outside the red cell near the opening. Bars represent 31 nm in panel a, 28 nm in b, 58 nm in c, 59 nm in d, 20 nm in e and 34 nm in f.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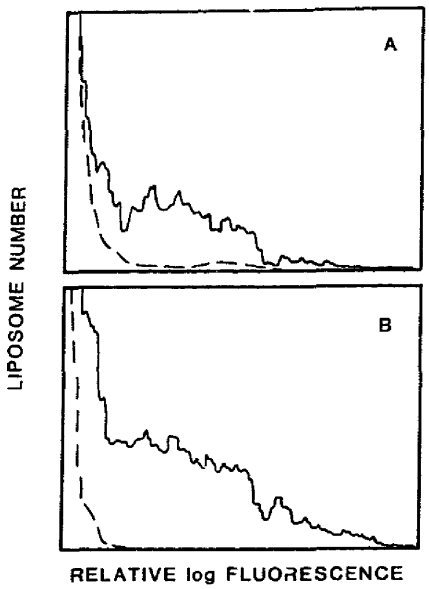

Fig. 6. Flow cytometry histograms of liposomes containing FITC-glycophorin in their membranes. (A) FITC-glycophorin was incubated at low pH with PC liposomes. After incubation with glycophorin monoclonal antibodies 10F7 and subsequently with Gam-IgG-Pe, the liposomes were analyzed by flow cytometry (———). (B) FITC-glycophorin was reconstituted into the liposome membrane during liposome formation. These liposomes were reacted with glycophorin antibodies 10F7 and Gam-IgG-Pe and analyzed by flow cytometry (- - - - -).

interaction mechanism, we studied the interaction of glycophorin with PC liposomes. Liposomes were incubated with glycophorin under the conditions used for the association of proteins with RBC membranes. After incubation with anti-glycophorin 10F7 monoclonal antibodies and with phycoerythrin-labeled secondary antibody, these liposomes showed a strong phycoerythrin fluorescence which was also detected by flow cytometry (Fig. 6A). Control liposomes, without glycophorin, but incubated with anti-glycophorin antibodies and phycoerythrin-labeled secondary antibodies, showed no fluorescence.

Glycophorin molecules reconstituted into PC liposomes by the method of MacDonald and MacDonald [16] span the lipid bilayer and the properties of these glycophorin-containing PC liposomes were well studied [20–24]. As shown in Fig. 6B, such liposomes, after incubation with antiglycophorin 10F7 antibodies and with the phycoerythrin-labeled secondary antibody, showed a fluorescence pattern similar to that of the liposomes with glycophorin associated with the bilayer by the low-pH method (Fig. 6A).

The experiments in Fig. 6 show the presence of glycophorin epitopes on the surface of liposomes, since the flow-cytometer measured single liposome fluorescence. When liposomes bearing low-pH-associated glycophorin or liposomes with glycophorin reconstituted into the membranes by the method of MacDonald and MacDonald [16] were incubated with higher concentrations of antibodies (antiglycophorin 10F7 and Gam-IgG-Pe), large liposome aggregates were formed (photographs not shown).

To compare FITC-glycophorin reconstituted into liposomes with FITC-glycophorin associated with liposomes after the low-pH incubation method, we performed polarization degree measurements. Prior to the measurements, both liposome populations were eluted through Sepharose 4B columns using PBS(7.4). Fluorescence microscope observations showed that both liposome populations were fluorescent, with comparable intensities. The polarization degrees, p, measured in PBS(7.4) of both liposome populations were the same: for reconstituted FITC-glycophorin into liposomes, $p = 0.329 \pm 0.05$ and for FITC-glycophorin associated with liposomes after the low-pH incubation, a p value of 0.334 ± 0.05 was obtained.

When a liposome suspension was added to a FITC-glycophorin solution at pH 4.7, a decrease in the FITC

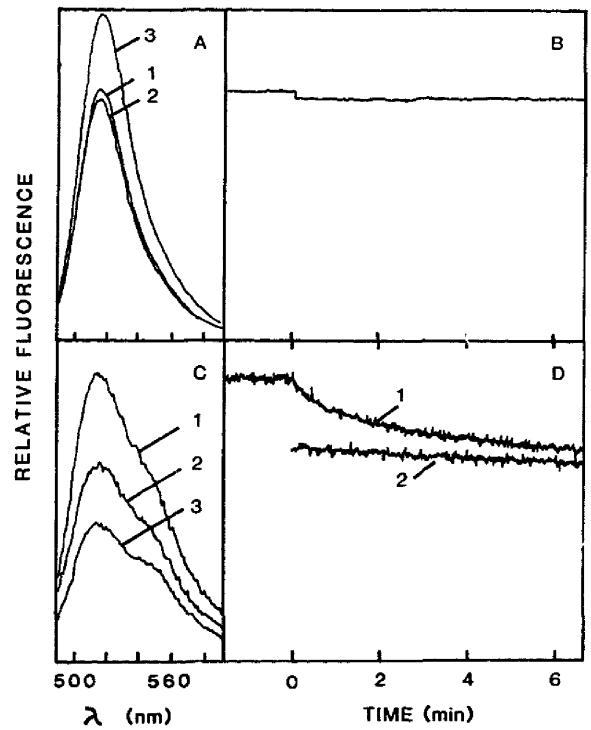

Fig. 7. Steady-state fluorescence changes during interaction of FITC-glycophorin with PC liposomes at pH 7.4 (A,B) and pH 4.7 (C,D). Panels A and C show emission spectra of FITC-glycophorin before (curve 1) and after (curve 2) interaction of FITC-glycophorin with PC liposomes. Curve 2 represents the emission spectrum at equilibrium. Curve 3 shows the changes in the emission spectra after reacting the samples with 1% (v/v) Triton X-100. Panels B and D show the time dependence of the changes at 518 nm of FITC-glycophorin fluorescence. At time zero, a liposome suspension was added to the glycophorin solutions. In panel D, curve 2 is the continuation of curve 1, thus, the recording time was 12 min.

fluorescence was observed (Fig. 7C,D). Addition of Triton X-100 to the sample after the reaction had reached equilibrium resulted in a further decrease of the fluorescence intensity at 518 nm and the stronger appearance of a shoulder in the region of 560 nm (Fig. 7C, curve 3). The behavior, together with the observation that the polarization degrees of reconstituted and low-pH-associated FITC-glycophorin are the same, indicate that the glycophorin molecule has changed its conformation during interaction with liposomes, i.e., entered a more hydrophobic environment (see Fig. 2 in Ref. 1) and is similar to the interaction of FITC-glycophorin with RBC (Fig. 3 in Ref. 1). Flow cytometry and fluorescence microscope observations of liposomes after low-pH incubation with FITC-glycophorin also showed a strong liposome-associated fluorescence (data not shown).

When liposomes were added to a FITC-glycophorin suspension at pH 7.4, after an initial decrease due to dilution effects, the FITC-fluorescence did not change with time, up to 30 min (Fig. 7B). Addition of Triton X-100 to the mixture resulted in an increase in FITC fluorescence (Fig. 7A, curve 2 to curve 3). The lack of association of FITC-glycophorin with liposomes at physiological pH was also documented by flow cytometry and fluorescence microscope experiments which showed that the liposomes, after incubation at pH 7.4 with FITC-glycophorin, were not fluorescent (data not shown).

Discussion

Our studies provide evidence that pH effects on the erythrocyte membrane trigger the low-pH interaction of membrane proteins with the erythrocyte membrane. The quantity of associated proteins began to be significant below pH 5, a pH region in which an abrupt change in the hemolysis properties of erythrocytes was also observed (see Fig. 1 and 2).

This change in hemolysis kinetics may be related to low-pH induced reversible aggregation of the membrane proteins of the erythrocyte ghosts in media between pH 5.5 and 4.5 [25–27]. The aggregation was related to the reduction in RBC membrane elasticity and increase in sphericity [28] due to spectrin aggregation, since extracted mixtures of spectrin and actin have the isoelectric point at pH 4.8 [29]. These effects could also explain our observation that the erythrocytes hemolyzed more easily below pH 4.7. The hemolysis time curves at low pH (Fig. 1) are similar to the hemolysis time curves of various detergents [30–32]; there is an apparent rapid initial rate of hemolysis which decreases after a few minutes to zero and then, after some time, hemolysis slowly increases and continues in a sigmoidal manner.

Direct evidence that low pH induces defects in the erythrocyte membrane was provided by electron microscope thin sections. As shown in Fig. 5. the defects appeared to originate as disturbances in the glycocalix (Fig. 5a) and develop into smaller (Fig. 5b) then larger (Fig. 5c,d,e,f) openings. For incubations at pH 4.7 of 1.5 min (Fig. 5e,f) the openings were not observed in more than 10% of erythrocytes in any experiment, in agreement with the 90° lightscattering hemolysis measurements (Fig. 1). Similar disturbances have been observed after lysoPC- or saponin-treatment of erythrocytes [33] and after early-stage hypotonic hemolysis [34]. Electron-dense materials appearing at the red cell exterior near openings (Fig. 5f, arrow) is an indication that loss of red blood cell components occurs through the observed defects. The interpretation agrees with the report of Baker [34], who documented the escape of hemoglobin from 20 nm openings in the RBC membrane during early stages of hypotonic hemolysis.

It should be emphasized that the openings reported here were observed during low-pH treatment of red cells in the absence of glycophorin. It is possible that in our electron microscopy experiments we do not detect the defects with which proteins may interact during the low-pH incubation. Rather, we detect these defects in a more mature form to which they develop in the absence of protein. In the presence of glycophorin at protecting concentrations (incubation for 1.5 min at pH 4.7), more than 99% of the red cells showed no membrane disturbance (data not shown). This result was in agreement with the observed protective effect of glycophorin against hemolysis (Figs. 3 and 4).

Our experiments with liposomes (Figs. 6 and 7) showed that glycophorin may associate at low pH with a lipid membrane which contains no proteins. During this association process, the changes monitored in FITC-glycophorin fluorescence (Fig. 7) are similar to those observed when FITC-glycophorin interacts with the erythrocyte membranes (Fig. 3 in Ref. 1). The polarization degrees of FITC-glycophorin reconstituted into liposomes by the method of MacDonald and MacDonald [16] and of glycophorin associated at low-pH to the liposome were the same; this observation, together with the fluorescence changes observed during FITC-glycophorin interaction with liposomes (pH 4.7, Fig. 7C,D), indicates that glycophorin molecules readjust conformation during low-pH interaction with liposomes. Protein aggregation on the liposome surface is unlikely, since multiple washings and washings performed after incubation with antibodies and secondary antibodies (Fig. 6) did not remove the membrane-associated proteins. This conclusion is also supported by the observation that passage through a Sepharose 4B column did not remove FITC-glycophorin associated to liposomes after the low-pH treatment. The polarization degree results and the recognition by monoclonal antibodies of liposome-associated glycophorin (Fig. 6) showed no difference between low-pH-associated glyco-

phorin and glycophorin reconstituted into liposome membranes. Since it has been shown that reconstituted glycophorin spans the liposome membranes [20–24], these results suggest that glycophorin entered the lipid bilayer after the low-pH incubation procedure.

The previously discussed effects of low pH on the erythrocytes and the observation that the pH-induced protein–membrane interaction takes place in erythrocytes and also in liposomes may be the result of the ability of protons to neutralize repulsive charges of the headgroups of phospholipids [35] and to reduce the thickness of the phospholipid hydration layer [36]. Protein-free lipid domains on the surface of erythrocytes, observed for erythrocyte ghosts [27], may favor such H^+–phospholipid interactions at low pH and the formation of 'point defects' which can act as interaction nucleation sites. The importance of defects in the organization of the lipid bilayer as loci for spontaneous incorporation of proteins into liposomes has been shown and studied [13–15]. In our case, the erythrocyte membrane morphological changes and the openings induced by low pH observed by electron microscopy (Fig. 5) may be mature forms of such defects. Recent studies visualized Ca^{2+}-induced domains in the bilayer of liposomes and erythrocyte ghosts, and it was shown that the domains depend on phospholipid composition and not on membrane proteins [37].

Although the emphasis in this study is on the effects of low pH on membranes, the low-pH effects on the proteins that interact with the membranes must not be neglected. Through protonation, the low pH may induce protein conformational changes which may expose more of the protein's hydrophobic regions to the environment, favoring in this way the interaction process. This was shown to be the case for purified G protein [17], bromelain-treated hemagglutinin of influenza virus [18], the E2 glycoprotein of Sindbis virus [19] or diphtheria toxin [3].

In conclusion, the tight association of proteins with erythrocyte membranes after incubation at low pH may be facilitated by a RBC membrane perturbed by H^+ ions. Creation of defects in the membrane which develop into openings in the absence of proteins in the incubation medium was shown. The observations made with liposomes indicate that in the mechanism of pH-dependent protein association with intact RBC membranes, an important component may be the interaction of the protein with the lipid component of the membrane of these cells.

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