BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Resolution of the paradox of red cell shape changes in low and high pH

Margaret M. Gedde [1,*], Eungyeong Yang [2], Wray H. Huestis

Department of Chemistry, Stanford University, Stanford, CA 94305, USA

Abstract

The molecular basis of cell shape regulation in acidic pH was investigated in human erythrocytes. Intact erythrocytes maintain normal shape in the cell pH range 6.3–7.9, but invaginate at lower pH values. However, consistent with predicted pH-dependent changes in the erythrocyte membrane skeleton, isolated erythrocyte membranes evaginate in acidic pH. Moreover, intact cells evaginate at pH greater than 7.9, but isolated membranes invaginate in this condition. Labeling with the hydrophobic, photoactivatable probe 5-[125I]iodonaphthyl-1-azide demonstrated pH-dependent hydrophobic insertion of an amphitropic protein into membranes of intact cells but not into isolated membranes. Based on molecular weight and on reconstitution experiments using stripped inside-out vesicles, the most likely candidate for the variably labeled protein is glyceraldehyde-3-phosphate dehydrogenase. Resealing of isolated membranes reconstituted both the shape changes and the hydrophobic labeling profile seen in intact cells. This observation appears to resolve the paradox of the contradictory pH dependence of shape changes of intact cells and isolated membranes. In intact erythrocytes, the demonstrated protein-membrane interaction would oppose pH-dependent shape effects of the spectrin membrane skeleton, stabilizing cell shape in moderately abnormal pH. Stabilization of erythrocyte shape in moderately acidic pH may prevent inappropriate red cell destruction in the spleen. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

The circulatory role of the human erythrocyte requires that its membrane be smooth and deformable.

Abbreviations: 125-INA, 5-[125I]iodonaphthyl-1-azide; G3PD, glyceraldehyde-3-phosphate dehydrogenase

* Corresponding author;
E-mail: mgedde@uclink4.berkeley.edu
[1] Present address: Department of Molecular and Cell Biology, 401 Barker Hall #3202, University of California, Berkeley, CA 94720-3202, USA. Fax: (510) 6435035,
E-mail: mgedde@uclink4.berkeley.edu
[2] Present address: SRI International, Menlo Park, CA 94025, USA.

This is true especially in the delayed circulation of the spleen, where blood pH drops as low as 6.8, and patrolling phagocytes remove cells unable to pass through narrow passages [1]. We have used the human erythrocyte, a highly amenable biological membrane model, to probe molecular mechanisms that regulate shape in low pH.

Low pH is predicted by well-developed mechanical models [2] to produce abnormal, spiked contours in circulating red cells through pH-dependent changes in the erythrocyte membrane skeleton, a thick, spectrin-based protein meshwork that lines the red cell inner membrane. The membrane skeleton behaves mechanically like an ionic gel, a flexible, three-dimensional, negatively charged structure sensitive to

changes in ionic strength and pH. Protonation of ionizable residues as pH is lowered is predicted to reduce charge repulsion within the gel and allow the elastic skeleton to contract, buckling the lipid bilayer physically coupled to it and producing outer surface spikes. In elevated pH the reverse process is expected to cause membrane invagination. Membrane curvature, like spectrin charge, would depend approximately linearly on pH [2]. The membrane skeleton is responsible for red cell deformability and elasticity and has analogs in a wide range of cell types [3].

Previous data on erythrocyte shape change presented a paradox: while intact cells and isolated membranes both change shape in low pH, isolated membranes form the predicted surface spikes [4–6], but intact cells develop surface invaginations [7–11]. Moreover, cells maintain normal shape in a wide pH range (6.3–7.9) [10], though isolated membrane shape is readily perturbed. It appeared that intact cells but not isolated membranes have a pH-responsive structure in addition to the membrane skeleton that modulates membrane shape.

In seeking to define the nature of this structure, we considered the following points. The best understood red cell shape changes occur by coupled-layer mechanisms where, because of tight apposition of the major membrane layers (the outer and inner lipid leaflets and the membrane skeleton), change in area of one layer induces bending of the other two layers [2,12–15], and significant bending can result from small (< 1%) change in layer area [13]. Intact cell shape change is rapid and reversible at room temperature [7–10,16], and it depends on cytoplasmic pH [11], implicating change in area of the inner lipid leaflet. However, phosphorylation and transbilayer distribution of membrane lipids play no detectable role [10].

On the other hand, study of certain cytoskeletal protein-membrane interactions suggests that some proteins function in both water-soluble cytoplasmic and hydrophobically embedded membrane forms [17]. Such proteins are termed 'amphitropic' to emphasize the reversibility of their movement between cytoplasmic and membrane compartments. Moreover, several peripheral and cytoplasmic erythrocyte proteins (spectrin [18,19], hemoglobin [20,21], band 4.1 [22], and actin [23,24]) insert hydrophobically

into anionic lipid-containing model membranes in a pH-dependent manner.

From these considerations we formulated the following hypothesis: pH-mediated shape changes of intact red cells are caused by pH-dependent, reversible association of a soluble red cell protein with the inner membrane hydrophobic phase. This hypothesis was examined using the hydrophobic, photoactivatable probe 5-[^{125}I]iodonaphthyl-1-azide (125-INA). Our investigations have uncovered a mechanism by which red cells appear to maintain normal shape in moderately acidic pH, a metabolic stress that confronts cells generally.

2. Experimental procedures

2.1. Preparation and pH equilibration of cells and membranes

Red cells in fresh citrated blood from healthy volunteers were washed in saline and suspended in phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 7.5 mM Na phosphate, 1 mM $MgSO_4$, 5 mM glucose, pH 7.4). Intact cells were equilibrated in buffers formulated to produce altered pH and normal range membrane potential and cell water [10].

Resealed red isotonic membranes ('resealed membranes') were prepared from hypotonically lysed erythrocyte membranes by addition of salt to isotonicity and 37°C incubation with Mg-ATP as described [25], a procedure that induces changes in both the membrane skeleton and the lipid bilayer [26]. Briefly, packed red cells were lysed in 40 vols. of Mg lysing buffer (14 mM HEPES, 2 mM $MgSO_4$, pH 7.4). Supernatant was removed, then salt and MgATP stocks were added to the following final concentrations: 10% membranes, 1% cytosol, 2 mM MgATP, 138 mM KCl, 6 mM NaCl, 2 mM $MgSO_4$, 10 mM HEPES, pH 7.4. Membranes were incubated 1 h at 37°C, then the inner pH of the resealed membranes was adjusted by equilibration in 1000 vols. of 145 mM KCl and 20 mM MES (pH 5.5 and 6.4), 20 mM HEPES (pH 7.2 and 7.9), or 20 mM CHES (pH 9.0).

Unsealed red hypotonic membranes ('red membranes') were prepared by lysing packed cells on ice in 10 vols. EDTA lysing buffer (10 mM HEPES,

1 mM EDTA, pH 7.4). pH was adjusted by resuspension in 20 vols. of 10 mM KCl and 5 mM MES (pH 5.5 and 6.4), HEPES (pH 7.2 and 7.9), or CHES (pH 9.0), for morphology measurements, or by dropwise addition of concentrated buffer to maintain 10% cytosol concentration for 125-INA labeling. Unsealed white hypotonic membranes ('white membranes') were prepared by lysing packed cells in 40 vols. EDTA lysing buffer on ice for 10 min and washing 2 times in 80 vols. EDTA lysing buffer. pH was adjusted by resuspension in 20 vols. of buffers used for red membrane pH equilibration.

2.2. Morphology assay

Morphology was scored under light microscopy for cells or phase contrast microscopy for membranes after fixation in 0.2% glutaraldehyde. Morphology was quantitated as morphological index, the average morphological score of at least 100 cells or membranes. Positive and negative values represent evagination and invagination, respectively, with discoid membranes scored as zero [16].

2.3. 125-INA labeling experiments

125-INA was prepared from 5-aminonaphthyl azide as described [27]. Cells (10% v/v) or membranes (33% v/v) equilibrated with 125-INA (final concentration < 0.2 μM) were irradiated at 305–400 nm for 40 min (cells) or 10 min (membranes). More membranes than cells were used to allow a shorter membrane irradiation time, which avoided membrane damage. Final membrane concentrations of probe are difficult to estimate, as the probe partitions readily into hemoglobin, and the amounts of hemoglobin present in the various preparations varied widely. Unreacted probe was quenched by addition of dithiothreitol (DTT). Unbound probe was removed by washing with and then without 1.25% bovine serum albumin in appropriate buffer. To prevent formation of high molecular weight complexes during preparation for electrophoresis, the following procedure was used: to 30 μl packed cells (or membranes) on ice were added 600 (60) μl of 5 mM Na phosphate, 2 mM EDTA, pH 7.6, then 100 (10) μl 70 mM N-ethylmaleimide, 14 mM EDTA, pH 7.0 and 300 (30) μl dye buffer (265 mM Tris-HCl, 8.4% SDS,

42% glycerol, 0.008% bromophenol blue, pH 6.8). Samples were boiled 2 min, 100 (10) μl 800 mM DTT was added, and samples were boiled 2 min further. Proteins were separated by SDS-PAGE in a Tris-glycine system. Autoradiography was performed using preflashed film.

3. Results

Hypotonically lysed and washed membranes ('white membranes') show the pH-dependent shape changes predicted from mechanical modeling of the membrane skeleton, as previously reported [4–6] and shown here (Fig. 1D), while intact cell shape dependence on pH is opposite that predicted and strikingly non-linear ([7–11] and Fig. 1A). Membranes that were hypotonically lysed but not washed ('red membranes') had the same shape dependence on pH as white membranes (Fig. 1C). In contrast, lysed membranes that were isotonically resealed ('resealed membranes') had the same pH-dependent shape response as intact cells (Figs. 1B and 2). Characteristics of the membrane preparations are shown in Table 1.

125-INA equilibrates into hydrophobic sites and, upon photoactivation of the azide to nitrene, covalently inserts into nearby carbon-carbon bonds [28]. This probe is expected to label only hydrophobic phase species: first, the vast majority of the probe equilibrates into the hydrophobic phase (partition coefficient $> 10^5$) [29]; second, trace probe present in the aqueous phase is predicted to be quenched immediately by water upon photoactivation to nitrene [28]. Lysozyme present in the supernatant of erythrocytes containing 125-INA did not incorporate probe upon irradiation (not shown). Moreover, consistent with previous reports [29], glutathione, an aqueous phase nitrene scavenger, did not alter the 125-INA labeling pattern of intact erythrocytes, while dithiothreitol, a scavenger that enters the hydrophobic phase, virtually eliminated 125-INA incorporation (not shown). Thus, no aqueous phase 125-INA labeling could be detected. However, cellular hydrophobic sites include not only the hydrophobic membrane core, but also the hydrophobic cores of some soluble proteins. Equilibration of 125-INA into certain soluble proteins was readily observed. 125-INA-loaded erythrocytes equilibrated with erythro-

cyte lysate (1.5 mM hemoglobin) or 3% (w/v) bovine serum albumin lost 18% of their 125-INA to the hemoglobin-containing solution and 98% to the albumin solution ($<2\%$ for buffer control). Therefore, the possibility that 125-INA incorporation was oc-

Fig. 1. Upon change in pH, membrane curvatures of intact erythrocytes and resealed erythrocyte membranes change in a direction opposite the changes in curvature of unsealed membranes. The figure shows morphological index as a function of cytoplasmic or inner pH for (A) intact erythrocytes, (B) resealed red isotonic membranes, (C) unsealed red hypotonic membranes, and (D) unsealed white hypotonic membranes. Positive and negative values of morphological index represent evagination and invagination, respectively. Plots are representative of two or more independent experiments. Starting morphology of membrane preparations varied, but the direction of shape pH dependence and the presence or absence of a plateau around neutral pH were always as shown. Error bar = S.D.
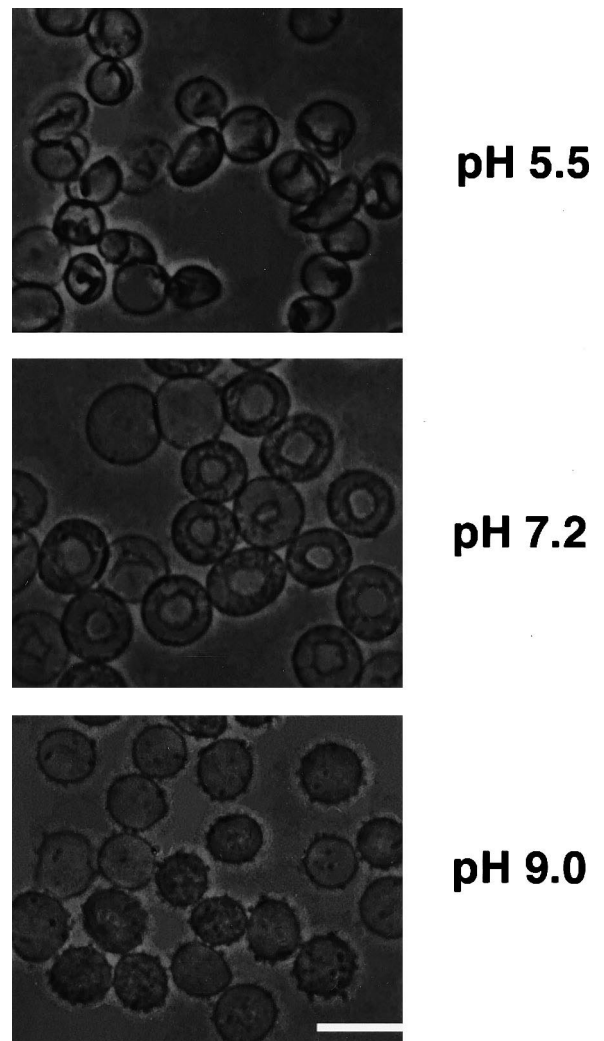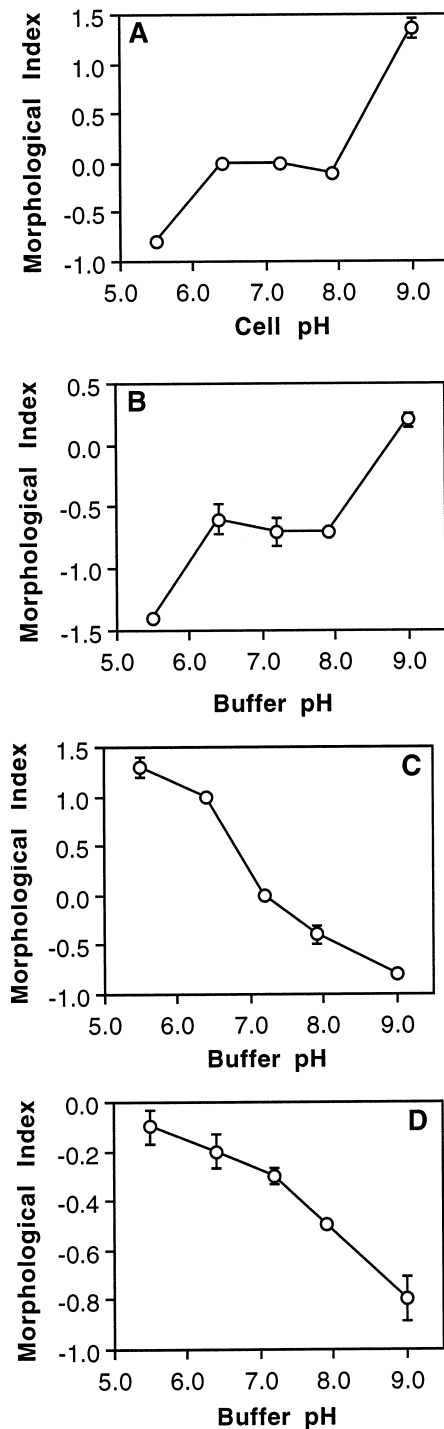←

Fig. 2. Resealed red membranes invaginate in low pH and evaginate in high pH. The shape changes parallel those of intact erythrocytes [10]. Size bar = 10 μm.

Table 1
Characteristics of erythrocyte membrane preparations

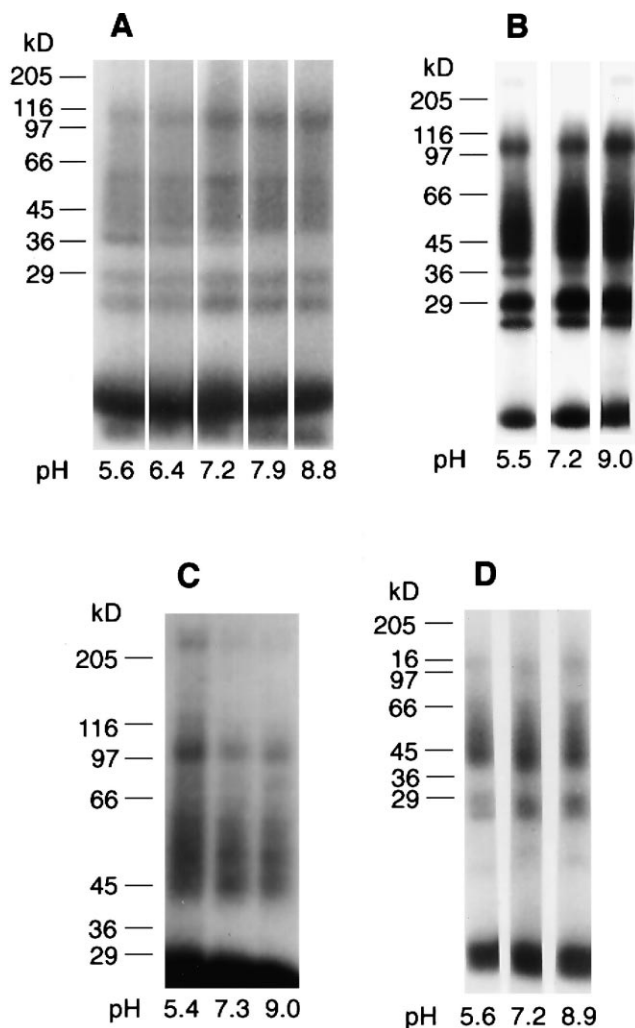|	Intact cells	Resealed membranes	Red membranes	White membranes
Cytosol concentration[a] (%)	100	2.5	10	0
Salt concentration	Isotonic	Isotonic	Low (25 mM)	Low (15 mM)
Membrane integrity	Unaltered	Post-hypotonic disruption and isotonic resealing	Post-hypotonic disruption	Post-hypotonic disruption

[a]Relative to that of intact cells.

Fig. 3. Incorporation of the hydrophobic probe 125-INA demonstrates pH-dependent, hydrophobic association of a 36 kDa protein with the membranes of intact or sealed, but not unsealed, erythrocyte membranes. Autoradiographs from SDS-PAGE of labeled (A) intact cells, (B) resealed membranes, (C) red membranes, and (D) white membranes are shown. Each autoradiograph is representative of two or more independent experiments.

curring in the hydrophobic core of an aqueous phase protein was considered in interpreting labeling results.

Intact erythrocytes incorporated 125-INA into electrophoretic zones corresponding to the integral membrane proteins Band 3, the red cell anion exchanger (90–100 kDa), Band 4.5, the glucose and amino acid transporters (40–60 kDa), and glycophorin C (around 30 kDa), as well as zones corresponding to the peripheral protein spectrin (260 and 225 kDa) and soluble hemoglobin tetramer (64 kDa), dimer (32 kDa), and monomer (16 kDa) (Fig. 3A). By 2D densitometry, probe incorporation was pH-independent for each of these proteins. However, labeling of a protein of apparent molecular weight 36 kDa was markedly increased at low pH and nearly absent at high pH, both visually (Fig. 3A) and when normalized to Band 3 labeling (Fig. 4). Red and white membranes exhibited no 36 kDa incorporation of 125-INA (Fig. 3C and 3D). In contrast, resealed membranes incorporated 125-INA at 36 kDa in the same pH-dependent manner as intact cells (Fig. 3B). Together with the shape results in Fig. 1, this finding supports the idea that this labeling pattern represents a pH-responsive mechanism operational in intact cells and reconstituted during the resealing process, one that counterbalances shape effects of the membrane skeleton.

Control experiments were designed to determine whether hydrophobic labeling of this species was taking place within the membrane or within a protein pocket. Intact, altered pH erythrocytes were suspended in cytosol-containing, normal ionic strength, altered pH supernatants and then labeled with 125-INA (Fig. 5). Since cytosolic proteins are not known to associate with the red cell outer surface, 125-INA labeling of supernatant 36 kDa protein would represent binding of probe to soluble protein. Cell and supernatant samples contained similar amounts of
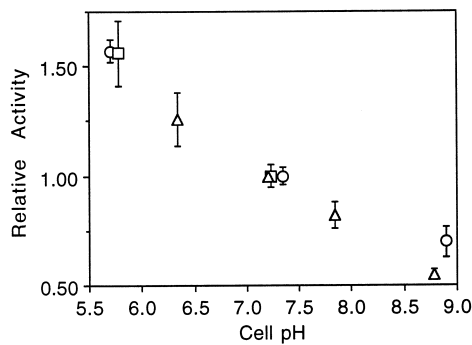
Fig. 4. Incorporation of 125-INA into a 36 kDa protein in intact erythrocytes decreases linearly with respect to cell pH. Intact cells prepared at altered cell pH, normal membrane potential, and normal cell water were labeled with 125-INA. Labeling relative to that of Band 3 was determined by 2D densitometry (Image, NIH). To compare experiments, labeling in each experiment was additionally normalized to the relative density of the normal pH sample and plotted against cell pH. Three experiments are shown. Error bar = S.D.

from incorporation of probe within the membrane, and not from artifactual incorporation into soluble protein.

4. Discussion

These data show that pH-dependent, hydrophobic labeling of a membrane-associated red cell protein correlates with ability of membranes to oppose pH-dependent mechanical effects of spectrin on membrane contour. The results offer a resolution to the paradox of the opposite pH dependences of intact and isolated erythrocyte membrane shapes, and suggest the following integrated model of how red cells maintain a smooth contour in moderately abnormal pH. Contraction of the membrane skeleton in low pH or expansion in high pH generates echinocytic or stomatocytic pressure on the membrane, respectively. Simultaneously, increased residency of an amphitropic protein in the hydrophobic phase at low pH, or decreased residency at high pH, produces stomatocytic or echinocytic pressure, respectively. In the cell pH range 6.3–7.9, opposing forces balance, stabilizing the normal discoid shape. Outside
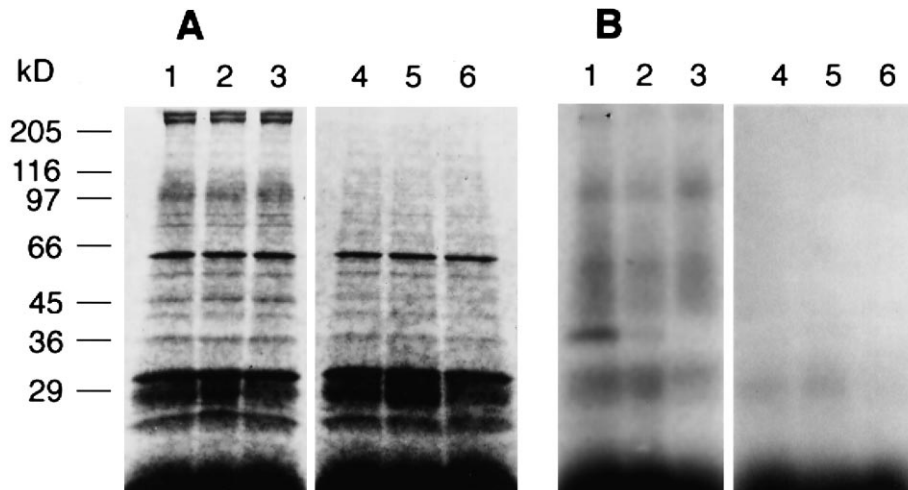
36 kDa protein by Coomassie stain (Fig. 5A). In contrast to the pH-dependent 36 kDa labeling observed in the cell samples, virtually no 36 kDa labeling was detected in the supernatant samples (Fig. 5B). Therefore, pH-dependent 36 kDa labeling in intact cells and resealed membranes must result

Fig. 5. pH-dependent 125-INA labeling of a 36 kDa red cell protein does not result from artifactual incorporation into soluble protein. To ask whether a 36 kDa species located in the soluble phase could incorporate 125-INA in a pH-dependent manner, 125-INA was extracted from intact, altered pH cells into cytosol-containing supernatants in the following manner. Supernatant from lysis of packed cells in 5 vols. of 5 mM sodium phosphate pH 8.0 was adjusted to normal ionic strength and pH 5.5, 7.2, or 9.0. 125-INA loaded intact cells with cytoplasm adjusted to the same pH values were resuspended in the supernatants and photoactivated, then cells and supernatants were separated. Proteins were resolved by SDS-PAGE. (A) Coomassie blue stain of whole cells (lanes 1, 2, 3) and cytosol (lanes 4, 5, 6) at pH 5.5 (lanes 1, 4), pH 7.2 (lanes 2, 5) and pH 9.0 (lanes 3, 6). (B) Autoradiograph of the gel in part A. 125-INA did not label soluble 36 kDa protein.

this pH range, shape effects of the protein-membrane interaction exceed those of spectrin volume changes and cause the stomatocytosis or echinocytosis observed in intact cells.

The only characterized 36 kDa protein in the human erythrocyte is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PD), also termed band 6 [30]. (An attractive candidate for the identity of the labeled 36 kDa species might be p36 (calpactin), a Ca^{2+}-dependent phospholipid/membrane-binding annexin family member and amphitropic protein [17], but normal human erythrocytes do not contain this protein [31].) In intact human erythrocytes, G3PD has been shown to be preferentially and specifically associated with the plasma membrane [32], but the chemical nature of the interaction is not known. In hypotonically lysed human erythrocyte membranes, and in inside-out membrane fragments prepared from them, G3PD is associated with the band 3 cytoplasmic tail by high affinity electrostatic bonds readily disrupted by high salt [33]. When inside-out vesicles were stripped of native G3PD, loaded with 125-INA, then incubated with purified exogenous G3PD, the added G3PD exhibited pH-dependent 125-INA labeling identical to that of the 36 kDa species in whole cells [34]. Therefore, G3PD is a likely candidate for the species promoting erythrocyte membrane stability in moderately altered pH. Which specific interactions would allow reversible partitioning of G3PD between soluble and hydrophobic phases is an open question. Covalent modification (fatty acid acylation) is not required for amphitropic proteins to coexist in the two phases [17]. Reversibility of amphitropic interactions suggests that low affinity bonds are involved; one possibility is that positive charges on soluble proteins are stabilized within hydrophobic sites by interaction with aromatic rings (cation-π interactions) [35]. An intriguing possibility is that changes in band 3 conformation might change the membrane orientation of G3PD bound to it; band 3 conformation changes have been proposed to underlie erythrocyte shape changes resulting from ionic strength changes and band 3 inhibitors [36].

For erythrocytes, a mechanism that maintains normal membrane curvature in low pH environments such as the spleen is probably of great adaptive value. However, all cells face potential buildup of acidic products of metabolism, and some cells appear actively to generate shifts in cell pH [37] which might disturb the balance of forces that sustains each cell's particular shape. Perhaps it is not coincidental that both spectrin-like proteins [3] and G3PD are virtually ubiquitous in eukaryotic cells. A G3PD-membrane interaction that compensates for pH-dependent perturbation by a spectrin-like protein may be a general mechanism for homeostasis of membrane contour in altered pH.

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