Characterization of Glycolytic Enzyme Interactions with Murine Erythrocyte Membranes in Wild type and Membrane Protein Knockout Mice

Short title for the running head: GLYCOLYTIC ENZYME INTERACTIONS WITH ERYTHROCYTE MEMBRANES

M. Estela Campanella¹, Haiyan Chu¹, Nancy J. Wandersee², Luanne L. Peters³, Narla Mohandas³, Diana M. Gilligan⁵, and Philip S. Low¹*

¹Department of Chemistry, Purdue University, West Lafayette, IN, 47907 USA; ²Department of Pediatrics and Children’s Research Institute, Medical College of Wisconsin, and Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI, USA; ³The Jackson Laboratory, Bar Harbor, Maine, USA, ⁴New York Blood Center, New York, NY 10021, USA. ⁵Puget Sound Blood Center, University of Washington School of Medicine, Seattle, WA 98104

- Address correspondence to: Philip S. Low, Department of Chemistry, Purdue University, West Lafayette, IN, 47907 USA. Tel. 765-494-5273 Fax 765-494-5272. E-mail: plow@purdue.edu

Scientific category: Red cells
Abstract
Previous research has shown that glycolytic enzymes (GEs) exist as multi-enzyme complexes on the inner surface of human erythrocyte membranes. Because GE binding sites have been mapped to sequences on the membrane protein, band 3, that are not conserved in other mammalian homologs, the question arose whether GEs can organize into complexes on other mammalian erythrocyte membranes. To address this, murine erythrocytes were stained with antibodies to glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphofructokinase, lactate dehydrogenase and pyruvate kinase and analyzed by confocal microscopy. GEs were found to localize to the membrane in oxygenated erythrocytes, but redistributed to the cytoplasm upon deoxygenation, as seen in human erythrocytes.

To identify membrane proteins involved in GE assembly, erythrocytes from mice lacking each of the major erythrocyte membrane proteins were examined for GE localization. GEs from band 3 knockout mice were not membrane associated, but distributed throughout the cytoplasm, regardless of erythrocyte oxygenation state. In contrast, erythrocytes from mice lacking α-spectrin, ankyrin, protein 4.2, protein 4.1, β-adducin or dematin headpiece exhibited GEs bound to the membrane. These data suggest that oxygenation-dependent assembly of GEs on the membrane could be a general phenomenon of mammalian erythrocytes and that stability of these interactions depends primarily on band 3.

Introduction
The glycolytic enzymes (GEs), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase, phosphofructokinase (PFK), lactate dehydrogenase (LDH) and pyruvate kinase (PK) have been shown to organize into multi-enzyme complexes on the inner surface of the human erythrocyte membrane. Because assembly of these complexes is sensitive to physiological stimuli such as band 3 phosphorylation and hemoglobin (Hb) deoxygenation, it has been suggested that regulation of GE assembly may serve an important biologic function, perhaps in regulation of glucose metabolism. Indeed, association of the complexes on the membrane has been observed to correlate with a shift in glucose metabolism from glycolysis to the pentose phosphate pathway. The specific docking sites of GEs on band 3 have been mapped to tandem similar sequences (6-DDYED-10 and 19-EEYED-23) near the NH2-terminus of the polypeptide. However, these NH2-terminal sequences are not well conserved in other mammalian species including the mouse, raising the question whether GE assembly into complexes is a feature of all mammalian red cells or a unique characteristic of human erythrocytes. Preliminary evidence in favor of GE binding to nonhuman erythrocyte membranes has come from staining data of Ercolani et al. and Weber et al. who show that antibodies to GAPDH label the membrane in both rat and mouse erythrocytes, respectively. However, no membrane staining of any other GEs has ever been reported in nonhuman erythrocytes, and the tyrosines with flanking acidic residues (Y8 and Y21) that are phosphorylated and in human band 3 and regulate GE association with the membrane are absent in murine band 3.

To determine the generality of GE assembly on erythrocyte membranes, we undertook to characterize the membrane binding sites, if any, of GAPDH, aldolase, PFK, LDH and PK on murine erythrocyte membranes. In the present study, we demonstrate that i) GEs are
indeed localized to the membrane in oxygenated murine erythrocytes, ii) GEs release from the membrane upon deoxygenation of the mouse red cells, iii) GEs are cytosolic in both oxygenated and deoxygenated erythrocytes from band 3 knockout mice, iv) GEs are displaced from the membrane by antibodies to the cytoplasmic domain of band 3 and by recombinant cytoplasmic domain of band 3 itself, and v) GEs are largely membrane localized in mature oxygenated cells from mice deficient in protein 4.2, β-adducin, protein 4.1, dematin headpiece domain, ankyrin (nb/nb) and α-spectrin (sph/sph). These data suggest that GE associations with erythrocyte membranes could represent a widespread phenomenon and that the stability of these interactions depends primarily on the presence of band 3.

Materials and Methods

Reagents
Poly-L-lysine and cold fish skin gelatin solutions were acquired from Sigma. Acrolein was purchased from Aldrich. Goat anti-rabbit muscle aldolase, PFK, LDH and PK were from Polysciences. Polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase and anti-cytoplasmic domain of band 3 (anti-cdb3) were generated in our own laboratory by immunization of rabbits against the purified human proteins according to standard procedures. Secondary antibodies, Rhodamine Red X and Cy2 labeled anti-rabbit and Rhodamine Red X, Cy2 and Cy5 anti-goat IgG were from Jackson Immunoresearch. Syto RNASelect green fluorescent cell stain was from Invitrogen and used as indicated for fixed cells by the manufacturer. The cytoplasmic domain of murine band 3 (cdb3, residues 1-398) was a recombinant protein expressed and purified in our lab and is described below.

Preparation of murine cdb3
A sense strand primer (5’TACATATGGGGACATCGGGACCAC-3’) and an antisense primer (5’-AAGCTTTCAAGATCCGCTGTGC-3’) were used both to amplify cDNA encoding murine cdb3 (residues 1-398) from murine full-length band 3 cDNA (Invitrogen) and to introduce a Nde I site and a Hind III restriction enzyme site. Using these restriction sites, murine cdb3 cDNA was inserted into a pT7-7 expression vector and protein was expressed in BL21(DE3) pLys S cells (Invitrogen). Recombinant cdb3 was then purified by the procedure used to purify recombinant human cdb3.

Preparation of cells
Mouse blood was collected either by cardiac puncture or retro-orbital sinus bleeding into heparinized tubes, shipped overnight on ice to Purdue University, and processed immediately upon arrival (no more than 24 h from the time of blood withdrawal). Samples were always accompanied by a travel control to assure that any possible stresses associated with shipping did not alter the results. Erythrocytes were washed twice in 330 mOsm phosphate buffered saline (PBS), pH 7.4, containing 5mM glucose. Processing for indirect immunofluorescence and evaluation of the effects of RBC deoxygenation were performed as previously described. Deoxygenation with argon was achieved by gently passing humidified argon across the erythrocyte suspension for 60 min. This deoxygenation caused the anticipated color change in the erythrocyte suspension, but
because the argon was humidified no change in suspension volume or hemolysis occurred. Oxygenation was achieved by simply washing the cells in oxygenated buffers. Deoxygenation with sodium dithionite was accomplished by evacuating 3mL of PBS-glucose solution in a 25mL vacuum flask for 10 min and then flowing humidified argon across the solution for 10 min. Ten millimolar dry sodium dithionite was then added to the deoxygenated solution, followed by addition of washed RBCs to a final hematocrit of 10%. Finally, argon was again gently blown across the sealed suspension for 3 min, and paraformaldehyde fixation was performed as previously described. Fixation of the deoxygenated cells prior to exposure to air was required to prevent re-binding of the enzymes to the membrane, which is a rapidly reversible process. Paraformaldehyde was used for initial fixation, because acrolein cannot fix cells in the absence of O2. This study received IACUC approval for the use of mice.

Confocal microscopy

Mouse erythrocytes were fixed, permeabilized and stained as previously described. Cells were subsequently attached to poly-L lysine coated coverslips (No 1.5 VWR) in humidified chambers for 15-20 minutes. Before observation cells were mounted on microscope slides using M800 Aqua Mount (Fisher Scientific Co) and sealed with nail polish. Samples were imaged with a Bio-Rad MRC1024 confocal microscope equipped with a 60x1.4 numerical aperture oil immersion lens using a 3.5 zoom factor. LaserSharp2000 software (Bio-Rad) was used in the acquisition of the images. Images were transformed into Tiff files using ThumbsPlus (Cerious software) and assembled into figures using Photoshop.

Results

Glycolytic enzymes are membrane localized in unperturbed oxygenated mouse erythrocytes.

We have demonstrated a reversible association of glycolytic enzymes (GEs) with the cytoplasmic domain of band 3 (cdb3) on the human erythrocyte membrane and we have shown that this association is regulated by erythrocyte oxygenation and band 3 phosphorylation. The sequences of human band 3 shown to be involved in this interaction, however, are not highly conserved in the mouse or in other mammalian species (Fig. 1); raising the possibility that membrane association of GEs might be a late evolutionary development. To explore whether GEs from murine erythrocytes are associated with the membrane, we fixed, permeabilized, and stained fresh murine erythrocytes with antibodies to GAPDH, aldolase, PFK, LDH and PK, and evaluated the GE distributions in stained whole cells. As seen in Fig. 2, all 5 GEs were found highly localized to the membrane in oxygenated murine erythrocytes.

Enzyme association is largely reversed upon erythrocyte deoxygenation

Previous studies have demonstrated that deoxygenated hemoglobin and GEs compete for overlapping binding sites at the NH2-terminus of band 3 in human erythrocytes, resulting in GE displacement from the membrane during red cell deoxygenation. To determine whether murine GEs are similarly responsive to Hb deoxygenation, fresh
murine erythrocytes were depleted of O2 prior to fixation and staining, and examined by confocal microscopy. As shown in Fig. 2, regardless of the method of deoxygenation, GEs translocate from the membrane to the cytoplasm upon oxygen removal. It should be noted that although the rate and mechanism of oxygen depletion are different for the dithionite and for the argon methods (dithionite consumes O2 by reacting with it, while argon depletes O2 from the atmosphere above the cell suspension, allowing the reversible dissociation of O2 from the cells in suspension18), neither method was seen to induce cell shape change or cell lysis (see bright field images of cells), suggesting that the GE translocations are not the result of damage to the erythrocyte membrane. Taken together, these findings imply that oxygenation-dependent assembly of GEs on the erythrocyte membrane occurs in mice as well as humans, despite significant divergence in their band 3 sequences.

Band 3 is the center of enzyme organization on the erythrocyte membrane
Although GAPDH, aldolase, PFK, LDH, and PK all translocate to the cytoplasm upon erythrocyte deoxygenation, only GAPDH, aldolase and PFK have been shown to bind human band 3 6. Indeed, in a previous study we were unable to document a direct interaction between band 3 and either PK or LDH 6. Because band 3 constitutes the only known binding site for deoxyHb on the membrane, displacement of PK and LDH during red cell deoxygenation was hypothesized to derive from their binding to proteins closely associated with band 3 1. However, the proximal membrane docking sites of LDH and PK have yet to be identified.

The availability of mice deficient in each of the major erythrocyte membrane proteins together with the knowledge that murine erythrocyte membranes bind GEs similar to human membranes now enables us to try to identify these putative proximal membrane binding sites. For this purpose, erythrocytes from band 3 knockout mice 9 were gently washed in oxygenated PBS and fixed with acrolein as described in Methods. After permeabilization, the fixed cells were stained as described in Materials and Methods and examined by confocal microscopy for both GE distribution and the presence of RNA (indicative of a reticulocyte). As seen in Fig. 3, none of the enzymes tested was found to localize on the membrane of band 3 knockout mice. While the vast majority of cells were admittedly reticulocytes (see Syto RNA Green stained panel in Fig. 3), even the few mature red cells that lacked RNA (lack of green staining) displayed no GE enrichment on the membrane. These data, therefore, suggest that band 3 is essential for proper assembly of GEs on the mature erythrocyte membrane.

To explore this hypothesis in greater detail, murine erythrocytes were resealed with an antibody to cdb3 and examined for displacement of GE from the membrane. Importantly, erythrocytes that were found to stain positive for entrapment of anti-cdb3 also revealed displacement of GE from the membrane. This is shown clearly in Figs. 4A and B where red cells that contain anti-cdb3 antibody in their cytosols (left panel; green staining in cytoplasm) are also seen to display strong anti-aldolase and anti-pyruvate kinase staining throughout their cytoplasms (left panel; red staining), suggesting that anti-cdb3 can block binding of GE to the membrane.
To corroborate this result further, fresh murine erythrocytes were resealed with a cloned cytoplasmic domain of murine band 3 (residues 1-398), and after fixation and permeabilization, similarly stained with antibodies to GAPDH, aldolase, LDH and PK. As shown in Fig. 5, cells that had entrapped the cloned mouse cdb3 display GEs distributed uniformly throughout their cytoplasms, indicating that free cdb3 can compete with endogenous cdb3 for assembling GEs. Finally, the same cloned mouse wild-type cdb3 was tested for its in vitro capacity to inhibit GAPDH activity, an assay commonly used to evaluate its binding affinity for cdb3. As shown in Supplemental Fig. 1, cdb3 inhibits the activity of the enzyme by about 70%. Taken together, we conclude that band 3 provides an important site for organization of GEs on murine red cells, just like it does on human red cells.

Requirement of other membrane proteins for assembly of GEs on the membrane.

To determine whether other membrane proteins might be similarly essential for GE organization on the membrane, erythrocytes from knockout mice for protein 4.2, β-adducin, protein 4.1, and dematin as well as red cells from mice containing spontaneous hypomorphic mutations leading to nearly quantitative deficiencies in ankyrin and α-spectrin were also examined. In contrast to membranes lacking band 3, GEs in these other defective erythrocytes still exhibit varying extents of membrane localization, suggesting that the essential components of their membrane binding sites remain intact (Fig 6 and 7, and Supplemental Fig. 2). Nevertheless, membrane localization is neither quantitative nor equivalent among all of the deficient membranes. Thus, membranes lacking protein 4.2, β-adducin, and protein 4.1 show little to moderate perturbation of GE binding, whereas membranes lacking ankyrin, dematin, and α-spectrin exhibit a moderate to substantial fraction of cells with aberrant GE distributions (Fig 6 and 7, and Supplemental Fig. 2). Importantly, most cells (but clearly not all cells) with cytosolic GEs also stain green with Syto RNA stain, suggesting many of these cells are reticulocytes rather than mature erythrocytes. Because reticulocytes still run the Krebs cycle and maintain much higher rates of glycolysis than mature erythrocytes, a different organization of GEs may be optimal.

Finally, it should not go unnoticed that the staining intensities of most of the free cytosolic GEs are invariably much greater than the staining intensities of their membrane-bound counterparts. In fact, in most cases, the fluorescence intensities of displaced enzymes were found to saturate the detector under the same conditions where the fluorescent signal from the membrane-bound enzymes was minimal. This differential in fluorescence intensity suggests that the epitopes recognized by anti-GE antibodies are largely occluded on membrane-bound GEs, but fully accessible upon displacement of the GEs into the cytoplasm. One possible hypothesis to explain this consistent observation is that GEs are so tightly associated into complexes on the membrane that accessibility of their epitopes is limited.

Discussion

Nucleated cells have highly compartmentalized interiors with enzyme pathways that are largely confined to organelles and cytosolic structures such as mitochondria, microtubules and microfilaments. The mammalian erythrocyte, in contrast, has none of the above cytosolic components, raising the question of whether organized metabolic
Pathways can be assembled in mammalian erythrocytes. Our recent observation that GEs are membrane bound in human erythrocytes may partially address this question, however, the weak homology between GE binding sites on human and murine homologs of band 3 has since raised concern that GE organization might be solely a late evolutionary development. We have now demonstrated that GEs are also membrane bound in oxygenated, healthy, murine erythrocytes, and that these associations are disassembled during normal deoxygenation in a manner similar to that seen in human erythrocytes. The observation that enzyme epitopes are largely inaccessible in membrane-bound GEs further suggests that interactions among GEs and other membrane components are tight, at least to the extent that they exclude antibodies. Indeed, data by Hoffman and others demonstrate that at least one type of GE complex on the erythrocyte membrane can sequester ATP and channel it directly to the membrane’s Na+/K+-ATPase. The GE complexes characterized by Hoffman and colleagues are also sufficiently tightly assembled that they can be shown to concentrate fluorescent ATP analogs on the membrane. Whether the glycolytic compartments characterized by these workers are similar to those observed in our study cannot be determined from the data.

Although many previous studies have concluded that band 3 constitutes a binding site for GEs on the membrane, no experiments have evaluated the possible participation of other membrane proteins in organization of GE assemblies. Using hypomorphic mutants and knockout mice, we have demonstrated that only the absence of band 3 leads to quantitative displacement of GEs from the membrane. While at least half of all erythrocytes in the sph/sph α-spectrin deficient mice displayed cytosolic GEs, the relatively few cells with little or no mRNA showed membrane binding of their GEs. Although other membrane proteins may still participate in membrane localization of GEs, these data argue that band 3 is the only membrane protein that is completely essential for GE assembly.

Finally, recent observations suggest that band 3 may exist in two distinct populations on the erythrocyte membrane. One population, associated with ankyrin, links β-spectrin to the membrane near the spectrin dimer↔tetramer association site. The other population attached to adducin, anchors the junctional complex to the membrane. To fully understand the architecture of the erythrocyte membrane, it will be important to determine whether one or both of these band 3 populations can organize GEs on the membrane.

Acknowledgements: This work was supported by grants NIH GM24417-29 (PSL) and NIH HL075714 (LLP). We greatly appreciate the generous gift of mouse blood from Athar Chishti (dematin head domain and band 3 null).

Authorship: M.E.C. performed experiments, analyzed data and wrote paper, H.C. performed experiments and helped analyze data, N.J.W, L.L.P., D.G, and M.N. provided mouse blood and edited the manuscript. PS.L. designed studies, analyzed data and helped write the paper.

Conflict of interest disclosure: The authors declare no competing financial interests.

References
Figure Legends:

**Fig. 1 Alignment of the human, dog, mouse, rat and cow NH2-terminus of band 3.**
Alignment of the first 65 amino acids of human, dog, mouse, rat and cow AE1 using the CLUSTALW program (http://align.genome.jp/). Asterisks (*) denote complete sequence conservation, whereas double and single dots indicate less conservation. The (^) symbols denote the two tyrosine phosphorylation sites at the NH2-terminus of human band 3.

**Fig. 2 GE staining in oxygenated and in deoxygenated mouse erythrocytes.** Confocal images of mouse erythrocytes following their staining for glycolytic enzymes under oxygenated (Air), deoxygenated with humidified argon (Argon), or deoxygenated with 10mM sodium dithionite (Dithionite) followed by humidified argon (see Methods).

**Fig. 3 Localization of GEs in oxygenated band 3 knockout erythrocytes.**
Confocal images of band 3 null erythrocytes stained for both RNA (to identify reticulocytes, green stain) and GEs (red stain), as indicated. A merger of both stains is shown in the third row (Overlay) and bright field images are displayed in the bottom row. Note that even the very few mature erythrocytes present (negative for SytoRNA stain, green color) do not show enzyme localization at the membrane.

**Fig. 4** Localization of glycolytic enzymes in murine erythrocytes resealed in the presence or absence of an antibody to the cytoplasmic domain of band 3. Freshly drawn murine erythrocytes were washed 3x to remove serum and buffy coat, and divided into two aliquots. One suspension of erythrocytes was lysed and resealed in the presence of rabbit polyclonal antibody raised against cdb3, according to methods in reference 1. The other suspension (control) was similarly lysed and resealed in the absence of any antibody.

After resealing, both erythrocyte preparations were similarly fixed with acrolein, permeabilized with Triton X-100 (see Methods), and stained with the desired antibodies. In panel a, anti-rabbit antibody conjugated to Cy2 (492/510) was used to identify cells containing entrapped anti-cdb3 antibody. In panel b, cells (lacking entrapped antibody) were first stained with the same rabbit anti-cdb3 antibody and then labeled with Cy2-conjugated anti-rabbit IgG. Membrane staining in this panel demonstrates the specificity of the antibody for band 3. Panels c and d were stained first with goat antibodies specific for either aldolase (Fig.4A) or pyruvate kinase (Fig. 4B) and then labeled with anti-goat antibody conjugated to Cy5 (650/670). Panels e and f are overlays of panels a and c, and b and d, respectively. Panels g and h show the bright field images of panels a, c, e, and b, d, f, respectively.

**Fig. 5** Localization of glycolytic enzymes in murine erythrocytes resealed in the presence of the cytoplasmic domain of murine band 3 (residues 1-398). Murine wild type cdb3 was dialyzed against 5mM potassium phosphate containing 160 mM sodium chloride, pH 7.4, and concentrated to 6.8mg/mL using a centricon-30 (Millipore). Freshly drawn mouse erythrocytes were washed in the above buffer and resuspended at 50% hematocrit in the same buffer containing mouse cdb3. The suspension was introduced into mini dialysis units, and lysed and resealed as described in reference 1. After resealing, erythrocytes were fixed, permeabilized and stained for GAPDH, aldolase, LDH and PK and visualized with secondary antibody conjugated with Cy2 (492/510). Control erythrocytes were lysed and resealed in the absence of cdb3, but otherwise treated identically. Glycolytic enzymes are displaced from the membrane in cells containing resealed murine cdb3.

**Fig. 6** Distribution of aldolase in erythrocytes from mice deficient in major erythrocyte membrane proteins. Intact erythrocytes from wild type mice and mice deficient in protein 4.2, β-adducin, protein 4.1, dematin headpiece domain, ankyrin (nb/nb), α-spectrin (sph/sph), and band 3 were fixed, permeabilized, and stained for aldolase and RNA, as described in Methods.
Fig. 7 Distribution of GAPDH in erythrocytes from mice deficient in major erythrocyte membrane proteins. All conditions are identical to those in Fig. 6, except anti-GAPDH was used to stain the cells rather than anti-aldolase.
Figure 1
Characterization of glycolytic enzyme interactions with murine erythrocyte membranes in wild type and membrane protein knockout mice

M. Estela Campanella, Haiyan Chu, Nancy J Wandersee, Luanne L Peters, Narla Mohandas, Diana M Gilligan and Philip S Low