Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane

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To characterize the location of glycolytic enzymes (GEs) in intact human erythrocytes, freshly drawn blood was fixed and stained with Abs to GAPDH, aldolase, phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), carbonic anhydrase II, Hb, and band 3 (AE1). Confocal microscopy revealed that in cells where band 3 displays its expected membrane staining and Hb is evenly distributed across the cytoplasm, GEs are largely limited to the membrane. Biochemical studies confirmed that the membrane binding sites for GAPDH, aldolase, and PFK reside on band 3, but related analyses demonstrate that sites for PK and LDH do not. Four lines of evidence demonstrate that the GEs are at least partially assembled into multimeric complexes near the NH_2 terminus of band 3. First, a mAb to residues 1–12 of band 3 displaces all of the above GEs from the membrane, including LDH and PK, which do not bind band 3. Second, tyrosine phosphorylation of the NH_2 terminus of band 3 (Y8 and Y21) reversibly releases all of the GEs from the membrane, including LDH and PK. Third, deoxygenation of RBCs dislodges all GEs from the membrane, consistent with the established ability of deoxyHb but not oxyHb to bind the NH_2 terminus of band 3. Fourth, a large increase in the accessibility of enzyme epitopes is observed upon dissociation of GEs from the membrane. We conclude, therefore, that GEs are organized into complexes on the membrane whose assembly is regulated by oxygenation and phosphorylation.

GAPDH | band 3 | AE1 | metabolon | compartmentalization

Because glycolytic enzymes (GEs) are largely found in the soluble fraction of whole-cell homogenates, most textbooks report that these enzymes diffuse freely in the cytoplasm. However, indications that GEs might actually be attached to cellular structures began to emerge as membranes isolated by hypotonic lysis of human RBCs were found to contain GAPDH (1, 2). Subsequent investigations of this enzyme's retention on the membrane revealed that GAPDH interacts specifically with an acidic sequence at the NH_2 terminus of band 3 and that this interaction exhibits high affinity ($K_d = 10^{-8}$ M) as long as the pH and ionic strength of the medium are kept low (3). Observations that aldolase (4) and phosphofructokinase (PFK) (5) also associate with the same NH_2-terminal sequence of band 3 then prompted interest in the possible assembly of a GE complex on the cytoplasmic domain of band 3 (6), and this interest subsequently led to investigations into whether the above enzymes are, in fact, membrane-bound in the intact cell.

Evidence supporting a physiologically relevant association between GEs and band 3 has derived from three types of experiments. First, a number of researchers have reported that GAPDH and aldolase are significantly inhibited upon association with the NH_2 terminus of band 3 and that this inhibition can be modulated by changes in pH, ionic strength, and the concentrations of various metabolic intermediates (3, 4). Second, rapid filtration experiments with saponin-lysed RBCs have suggested that between one-third and two-thirds of the above enzymes are immobilized (i.e., membrane-bound) at the instant of cell lysis (7, 8). Finally, resealing of a mAb fragment (F_{ab}) specific for the GE binding site on band 3 into freshly drawn RBCs has been found to accelerate glycolysis (presumably by displacing the enzymes from their inhibitory sites on band 3), whereas introduction of the inhibitory band 3 peptide into similar red cells causes a major reduction in glycolytic flux (9).

Evidence against the claim that certain GEs might be membrane bound *in vivo* has also been based on multiple observations. First, it has repeatedly been pointed out that an association between band 3 and the above GEs does not occur at physiological ionic strength and pH (10, 11). The fact that GAPDH, aldolase, and PFK are retained on membranes after hypotonic lysis was simply dismissed as an artifact of nonspecific ionic interactions between the acidic NH_2 terminus of band 3 (of the first 33 amino acids, 18 are acidic, none are basic, and the NH_2 terminus is blocked) and basic GEs (the pI values of most GEs are >8). Second, several species of mammalian RBCs do not retain GAPDH (nor presumably any other GE) on their membranes, suggesting that the putative interaction is not of widespread importance (12, 13). Indeed, the GE binding sequence at the NH_2 terminus of band 3 is one of the least conserved regions of the polypeptide (13–15). Third, despite significant differences in GAPDH retention in hypotonically prepared membranes, all mammalian RBCs exhibit similar glycolytic rates (16), suggesting that any differences seen in membrane association have no functional consequence. Fourth, more recent studies employing a modified version of the aforementioned rapid filtration technique report no evidence for membrane binding of GEs when proper adjustment for the kinetics of hemolysis is made (10). And finally, mathematical modeling of glycolytic fluxes in human RBCs does not require consideration of any inhibitory band 3–enzyme complexes to achieve a good fit with the experimental data (16). In fact, the catalytic capacity of GAPDH in human RBCs is so high that inhibition of >90% of the enzyme through association with band 3 would not be expected to affect the glycolytic flux (16). Thus, given the absence of compelling experimental evidence for a band 3 interaction *in vivo* and the lack of a strong theoretical argument for its existence, the concept of a membrane-localized compartment of GEs has been dismissed by its opponents as an experimental artifact (16, 17).

Three unrelated observations have recently rekindled our interest in the question of whether GEs are membrane-bound and organized into complexes in human RBCs. First, molecular crowding effects on macromolecular interactions in intact RBCs have been evaluated and shown to be sufficiently high to significantly enhance protein interactions that might display no apparent affinity under dilute laboratory conditions (18, 19). Based on these considerations, enzyme–band 3 associations that cannot be sustained at physiological pH and ionic strength *in vitro* can still be argued to exist *in vivo*. Second, Rogalski *et al.* (20) and Alper and colleagues (21) have recently thin-sectioned glutaraldehyde-fixed whole RBCs and shown that immunostaining for GAPDH is concentrated at the membrane surface. And finally, Hoffman and colleagues (22) have provided evidence for a membrane-bound compartment of adenine

Abbreviations: PFK, phosphofructokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; GE, glycolytic enzyme; cdb3, cytoplasmic domain of band 3.

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nucleotides that serves to channel ATP directly from its glycolytic source to ion pumps that use it. These latter data indicating a biological function for assembly of enzymes on the membrane, and together with other data demonstrating an association between the Na^+/K^+-ATPase and band 3 (23, 24), suggest that an interaction between band 3 and GEs might be physiologically relevant. Because there has been no attempt to resolve the question of enzyme localization in intact RBCs with confocal microscopy, we have developed a method for rapidly fixing freshly drawn human RBCs in a manner that preserves the integrity of intracellular complexes. We provide evidence here that GEs are not only organized into a complex on the human RBC membrane but that assembly and disassembly of the complex is regulated by physiologically relevant stimuli.

Materials and Methods

The reagents we used were poly(L-lysine), PMSF, sodium orthovanadate, H_2O_2, and cold fish-skin-gelatin solution from Sigma; acrolein and diisopropylfluorophosphate from Aldrich; staurosporine from Calbiochem; nitrocellulose membranes, chemiluminescence reagents, and Triton X-100 from Pierce; goat Abs against rabbit muscle aldolase, lactate dehydrogenase (LDH), pyruvate kinase (PK), PFK, and rabbit anti-bovine carbonic anhydrase II from Polysciences; and rabbit anti-human RBC GAPDH and anti-Hb were produced locally by standard methods. The anti-Hb IgG was affinity-purified by using Hb covalently bound to Affi-gel-10 from Bio-Rad. A mAb against the NH_2-terminal 12 residues of band 3 was generated and characterized by Willardson *et al*. (25). Anti-phosphotyrosine Ab (PY99) was from Santa Cruz Biotechnology. The secondary Abs purchased from Jackson ImmunoResearch were donkey $F(ab')_2$ fragments of anti-goat, anti-mouse, and anti-rabbit conjugated to either rhodamine red-X or cyanine Cy2 and donkey $F(ab')_2$ fragments of anti-mouse or anti-rabbit conjugated to Cy5.

Preparation of Cells for Immunofluorescence. Blood was drawn from healthy volunteers with their informed consent and pelleted at $1,000 \times g$ for 10 min at room temperature. After removal of the buffy coat, RBCs were pelleted and washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Ka_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 5 mM glucose and then fixed for 5 min in 0.5% acrolein in PBS. Cells were rinsed three times then permeabilized in PBS containing 0.1M glycine (rinsing buffer) plus 0.1% Triton X-100 for 5 min and again rinsed 3X in rinsing buffer. To ensure complete neutralization of unreacted aldehydes, cells were then incubated in rinsing buffer at room temperature for 30 min. After incubation, all nonspecific binding was blocked by incubation again for >60 min in blocking buffer (PBS containing 0.05 mM glycine, 0.2% fish skin gelatin and 0.05% sodium azide). Staining of fixed, permeabilized RBCs was performed by using Abs diluted in blocking buffer. After labeling, resuspended RBCs were allowed to attach to cover slips coated with polylysine, and the cover slips were mounted by using Aqua-Mount (Lerner Laboratories, New Haven, CT). Samples were imaged with a Bio-Rad MRC1024 (Bio-Rad) confocal microscope equipped with a 60×1.4 numerical aperture oil immersion lens.

When desired, a mAb to the NH_2 terminus of band 3 was resealed at 37°C into freshly drawn RBCs according to the method of Scott *et al*. (26), except that the cells were lysed at 50% hematocrit for 80 min and resealed in PBS plus 5 mM glucose for 60 min. Before fixation, the resealed cells were allowed to recuperate for 20 min in PBS containing 5 mM glucose and 0.2% BSA.

Evaluation of the Effects of RBC Deoxygenation on Enzyme Localization. RBCs suspended at 10% hematocrit in 3 ml of PBS-glucose in a 25-ml vacuum flask were gently evacuated for 10 min, after which humidified argon was blown gently across the suspension for 50 min. Paraformaldehyde (1% final concentration) was then added to the flask and allowed to fix the cells for 20 min (initial fixation with paraformaldehyde is necessary, because acrolein cannot fix cells in the absence of O_2). Paraformaldehyde-fixed cells were then centrifuged and resuspended in acrolein (0.5% prepared in PBS) before permeabilization and staining as described above.

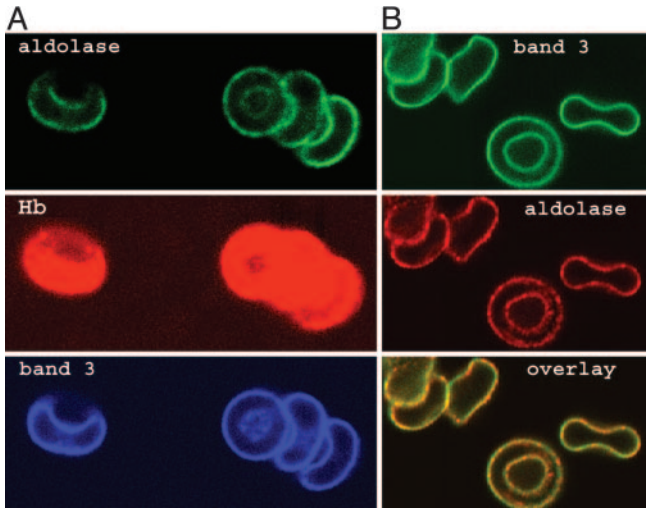

Fig. 1. Confocal microscopy of fresh human RBCs stained for aldolase, Hb, and band 3. Freshly drawn RBCs were processed as described in *Materials and Methods*, where the same sample was stained initially for Hb, then aldolase and finally band 3 (*A*) or initially for band 3 and then aldolase (*B*). (*B*) An overlay of aldolase and band 3 is provided to demonstrate the coincidence of their staining patterns.

Phosphorylation, Electrophoresis, and Immunoblotting of RBCs. Washed and pelleted RBCs in 10 mM Tris·HCl, pH 7.4, containing 150 mM NaCl and 2 mM $MgCl_2$ were resuspended at 10% hematocrit. Samples were initially incubated for 30 min at 37°C in buffer alone or buffer supplemented with 3 μM staurosporine. Pervanadate (final concentration, 15 μM H_2O_2/5 μM Na^+ orthovanadate) was then added to all samples except controls, and samples were further incubated for 15 min at 37°C. Pelleted cells were either processed immediately for immunofluorescence microscopy or hemolysed in hypotonic buffer (5 mM Na_2HPO_4, pH 8.0/1 mM DIFP/0.1 mM PMSF/1 mM Na^+ orthovanadate/0.02% NaN_3) before electrophoresis on 10% polyacrylamide gels and immunoblotting by using Bio-Rad protocols and chemiluminescence reagents.

Results

GE Localization in Unstimulated RBCs. The controversy over whether GEs are associated on the human RBC membrane *in vivo* has recently evolved into a debate over which experiments have been conducted under physiologically relevant conditions. Therefore, to characterize the location of GEs under the most physiological conditions achievable, we have developed a method for rapidly fixing freshly drawn human RBCs and analyzing the localization of GEs in the intact cells by confocal microscopy (see *Materials and Methods*). Working under these conditions, we have invariably obtained fixed cells that stain for band 3 on the membrane and Hb throughout the cytoplasm (Fig. 1*A*). More importantly, staining of the same cells for GEs has demonstrated their distinct membrane localization, with little enzyme staining in the cytoplasm. Evidence that restriction of the anti-aldolase and anti-band 3 Abs to the cell periphery is not a trivial consequence of their inability to penetrate the highly cross-linked Hb-dense cytoplasm can be gleaned from consideration of the triply labeled cells in Fig. 1*A*. Thus, in the same fixed RBCs where anti-Hb Abs experience no barriers to permeation of the cell interior, staining for aldolase and band 3 is limited

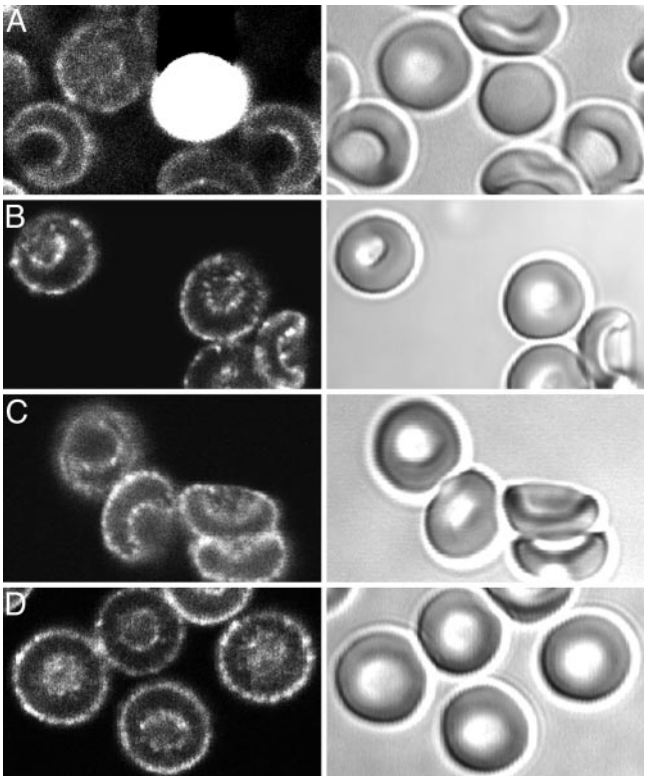

Fig. 2. Confocal immunofluorescence (*Left*) and corresponding brightfield (*Right*) images of freshly drawn RBCs stained with Abs for GAPDH (*A*), PFK (*B*), LDH (*C*), and PK (*D*). Note that the only cell that displays strong cytoplasmic staining has spherocytic morphology. Additional micrographs and numerical analyses of cells with membrane localized enzymes are provided in Fig. 6 and Table 2.

to the membrane. However, caution must be exercised in using other fixation protocols, because more aggressive fixation was found to result in a Hb staining pattern also restricted to the cell periphery (data not shown).

To further demonstrate that the mild fixation protocol does not introduce unwanted staining artifacts, aldolase localization was compared with band 3 localization in the same cells by using a mAb to the cytoplasmic domain of band 3 (cdb3). As shown in Fig. 1*B*, aldolase clearly colocalizes with band 3, suggesting that the enzyme indeed resides on the membrane in intact minimally perturbed RBCs. Importantly, similar results were also obtained when the RBCs were rapidly fixed in freshly drawn whole blood, suggesting that the removal of the plasma does not induce membrane localization (data not shown). Indeed, the only reproducible differences observed between band 3 and aldolase staining have been the somewhat punctate or particulate distribution of aldolase (and the other GEs) compared to the even staining of band 3 (see Figs. 1–5) and the presence of some enzyme staining in the cytoplasm compared with the absence of band 3 staining off the membrane.

To evaluate whether other GEs might also assemble on the cytoplasmic surface of the human RBC membrane, similarly fixed cells were stained for GAPDH, PFK, LDH, and PK. As documented in Figs. 2, in virtually all cases, membrane localization is observed, suggesting that none of the investigated enzymes is randomly distributed throughout the cytosol in healthy oxygenated cells. Although a few cells do display a cytosolic concentration of enzymes (see Fig. 2*A*), these cells have invariably been found to constitute a small fraction of the total population (<2%; see also Figs. 6–8 and Table 2, which are published as supporting information on the PNAS web site). In fact, in those rare cases for which cytosolic staining is observed, the cells appear spherocytic or otherwise abnormal (see brightfield images), suggesting that they might be dehydrated, senescent, or otherwise unhealthy. These results indicate that the GEs examined are membrane-bound in unstimulated, healthy RBCs.

Evaluation of LDH and PK Interactions with the Cytoplasmic Domain of the Membrane-Spanning Protein band 3. As noted in the Introduction, much previous work has implicated the extreme NH_2 terminus of band 3 as a likely binding site for GAPDH, aldolase, and PFK (3–5). Thus, aldolase and GAPDH were shown to be inhibited by sequences in this region of band 3 (3, 4), and the sensitivity of PFK to physiological modulators was found to be altered upon association with the NH_2 terminus of band 3 (5). Noting that similar studies had not been conducted with LDH or PK, we decided to explore whether these enzymes might also exhibit an affinity for cdb3. Borrowing from the earlier methods of Steck and colleagues (3, 4), we confirmed that the catalytic activities of GAPDH and aldolase are strongly inhibited upon addition of cdb3 (Table 1), suggesting that band 3 might constitute the membrane binding site of these enzymes in the intact cell. However, contrary to expectations, neither LDH nor PK displayed any perturbation of catalytic function in the presence of cdb3 (Table 1).

Table 1. Effect of saturating concentrations of band 3 constructs on the catalytic activities of glycolytic enzymes

	GAPDH activity, %	Aldolase activity, %	PFK activity, %	LDH activity, %	PK activity, %
No band 3	100	100	100	100	100
Wild-type cdb3*	7 ± 1.8	35 ± 0.5	21 ± 0.7	102 ± 4.3	107 ± 8.7
COOH terminus of band3†	105 ± 7.9	99 ± 2.9	92 ± 16.5	96 ± 4.1	97 ± 7.7
Kidney cdb3‡	105 ± 9.6	100 ± 4.3	98 ± 0.2	98 ± 7.3	106 ± 7.1

GAPDH activity was assayed according to ref. 3, except that the reaction cuvette contained 10 mM imidazole acetate (pH 7.0), 0.1 mM EDTA, 0.05 mM sodium arsenate, 1 mM Pi, 0.3 mM NAD, and 0.05 mM glyceraldehyde-3-P. Aldolase activity was assayed according to ref. 37. PFK activity was assayed according to ref. 5, except that the cuvette contained 10 mM Tris·HCl (pH 7.0), 0.2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NADH, 1 mM Pi, 1 mM ATP, 1 mM fructose-6-P, 0.4 units of aldolase, 2.4 units of triose phosphate isomerase, and 0.4 units of α-glycerophosphate dehydrogenase. LDH and PK activity was assayed according to ref. 38, except that the curvette contained 10 mM phosphate (pH 7.5), 5 mM pyruvate, and 0.2 mM NADH.

*Residues 1–379 of human RBC band 3.

†Residues 872–911 at the extreme COOH terminus band 3, expressed as a fusion protein with GST attached to the peptide's NH_2 terminus.

‡Residues 66–379 of human RBC band 3 (comprising the cytoplasmic domain of kidney band 3), modified with a His_6 tag at its COOH terminus.

To further explore whether band 3 might provide the unidentified docking site for LDH or PK on the membrane, we then evaluated whether LDH or PK might bind to an exposed region of the COOH terminus of band 3. Thus, as alluded to above (3, 4), aldolase and GAPDH probably associate with one of two tandem homologous sequences at the extreme NH_2 terminus of band 3, compromising residues 5-QDDYED-10 and 18-QEEYED-23. Because a third homologous sequence exists at the extreme COOH terminus of band 3 (residues 901-RDEYDE-906), we hypothesized that the membrane docking site for LDH or PK might reside in this sequence. However, the COOH terminus of band 3 was also found to have no impact on the catalytic activities of these enzymes.

Recognizing that LDH and PK might associate with band 3 without experiencing any change in their catalytic functions, we also looked for a band 3 association by two other methods. First, the ability of cdb3 to inhibit GAPDH was examined in the presence and absence of increasing concentrations of LDH and PK. We reasoned that even if the catalytic activities of LDH and PK were unperturbed by binding to cdb3, if binding to cdb3 nevertheless occurred, LDH or PK might compete with GAPDH and prevent its inhibition by cdb3. However, addition of neither LDH nor PK to the GAPDH assay exerted any impact on the inhibition of GAPDH by cdb3 (data not shown).

Finally, to directly measure association of cdb3 with LDH and PK, cdb3 was immobilized onto SulfoLink beads (Pierce) and the immobilized polypeptide was allowed to associate with the desired GE. Although GAPDH was readily found to copellet with cdb3, neither LDH nor PK demonstrated any association with the same beads (data not shown). Therefore, we conclude that the binding site of LDH and PK on the human RBC membrane does not reside on band 3.

Evaluation of Enzyme Distribution Along the Membrane Surface. As noted above, comparison of the staining patterns for band 3 and the GEs in the same field of RBCs suggests that membrane staining for band 3 is always smooth whereas staining for GEs can be measurably punctate. To determine whether the interrupted enzyme staining might be due to a handling or fixation artifact, several other methods of sample processing were also developed and examined. Regardless of whether the RBCs were fixed in whole blood immediately after phlebotomy, washed free of plasma before fixation, or washed and allowed to adhere to a glass slide before fixation, a similar punctate distribution was observed (data not shown). Although uneven Ab accessibility to evenly distributed enzyme epitopes could explain the punctate distribution, the fact that similar punctate staining patterns are seen with polyclonal and monoclonal antienzyme Abs (data not shown) would seem to argue against this interpretation. Rather, we speculate that a fraction of the enzymes might be organized into larger complexes and that these complexes might appear as brighter fluorescent intensities on the membrane.

Direct Evidence That the GE Complexes on the Human RBC Membrane Are Organized Around band 3. In an effort to examine the possible participation of band 3 in organizing the assembly of GEs on the membrane, we undertook to examine the impact of known perturbants of band 3–enzyme interactions on the distribution of GEs in the cell. For the initial site-specific perturbation, we exploited the method of Scott *et al.* (26) to reseal a mAb to the GE binding site at the NH_2 terminus of band 3 into freshly drawn red cells under conditions where little loss of Hb and endogenous enzymes is known to occur (see *Materials and Methods*). As shown in Fig. 3 (see also Fig. 7), all cells that successfully entrapped the anti-band 3 Ab (see cells staining positive for anti-band 3) showed strong displacement of the GEs. In contrast, cells that failed to take up Ab during the cell lysis/resealing procedure also failed to show any enzyme displacement. Surprisingly, although LDH and PK are not thought to bind band 3, they are still displaced from the membrane by the

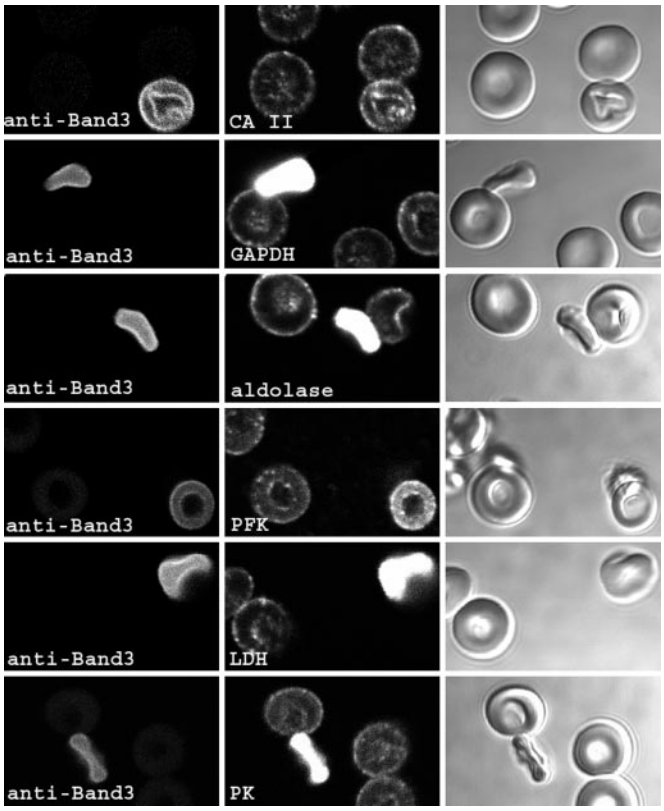

Fig. 3. Impact of a mAb to the NH_2 terminus of band 3 upon GE binding to the red-cell membrane. Washed RBCs were lysed and resealed in the presence of a mAb to residues 1–12 of band 3, as described in *Materials and Methods*. Entrapment of anti-band 3 (*Left*) was visualized by staining with donkey anti-mouse conjugated with Cy2, whereas enzyme localization in the same field of cells (*Center*) was visualized as in Fig. 2. (*Right*) Micrographs displaying a brightfield image of the same cells. Note that enzyme displacement from the membrane is seen only in cells that contain entrapped mAb. See Fig. 7 for additional micrographs.

Ab that recognizes band 3. To demonstrate that this unanticipated displacement of LDH and PK was not a simple consequence of a nonspecific perturbation of band 3, the distribution of carbonic anhydrase II, an enzyme known to associate specifically with the COOH terminus of band 3, was also examined (27). As seen in Fig. 3, RBCs containing the Ab to the NH_2 terminus of band 3 showed no displacement of carbonic anhydrase II from the COOH terminus of band 3. These data, therefore, suggest that the observed displacement of LDH and PK by the Ab to the NH_2 terminus of band 3 occurs because of the enzyme's proximity to or association with the other GEs that bind band 3 and not because of some nonspecific membrane perturbation.

A second, more physiological perturbation of the GE binding site at the NH_2 terminus of band 3 should occur during RBC deoxygenation. Thus, deoxyHb displays high affinity for the NH_2 terminus of band 3 and, in fact, completely occludes the GE binding site by encircling it within its 2,3-bisphosphate binding cavity in the center of the molecule (28). Because this 2,3-bisphosphate binding cavity closes upon Hb oxygenation, oxyHb exhibits comparatively little affinity for band 3 (28). Thus, if the association between band 3 and the GEs is physiological, one would expect to observe at least partial displacement of GEs upon deoxygenation. As shown in Fig. 4, this result is indeed observed, not only for the enzymes known to associate with the NH_2 terminus of band 3 but also for the enzymes (PK and LDH) explicitly shown not to bind to this sequence. Because this oxygenation-dependent displacement is seen in intact unperturbed cells, we conclude that all GEs examined bind at or

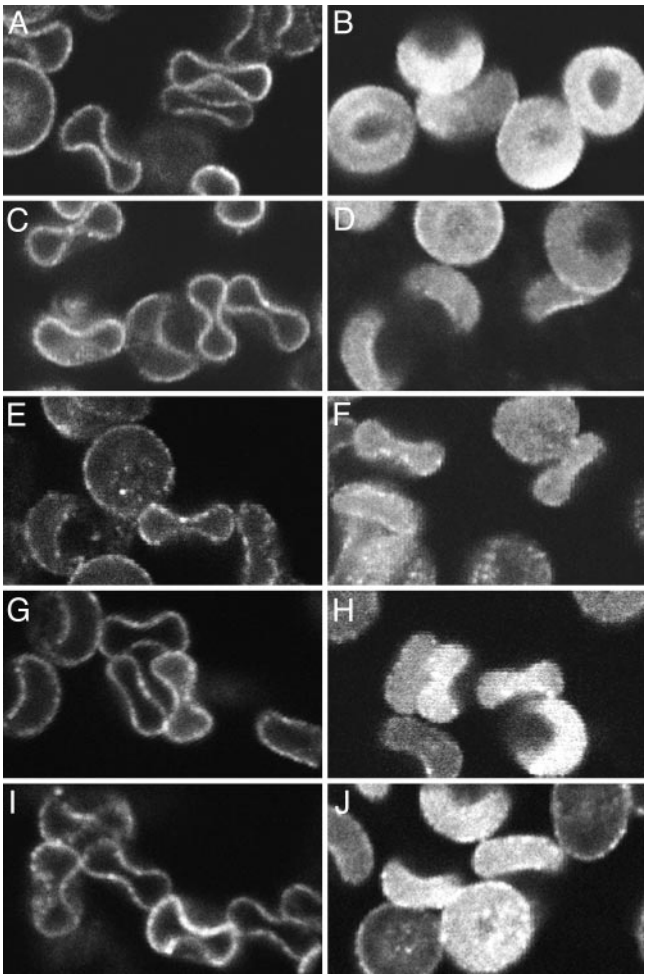

Fig. 4. Effect of deoxygenation on the distribution of GEs in intact RBCs. Washed cells were deoxygenated by evacuation under argon and then fixed and stained as described in *Materials and Methods*. (*A*, *C*, *E*, *G*, and *I*) Samples equilibrated in air. (*B*, *D*, *F*, *H*, and *J*) Deoxygenated samples equilibrated in argon. Cells are stained for GAPDH (*A* and *B*), aldolase (*C* and *D*), PFK (*E* and *F*), LDH (*G* and *H*), and PK (*I* and *J*).

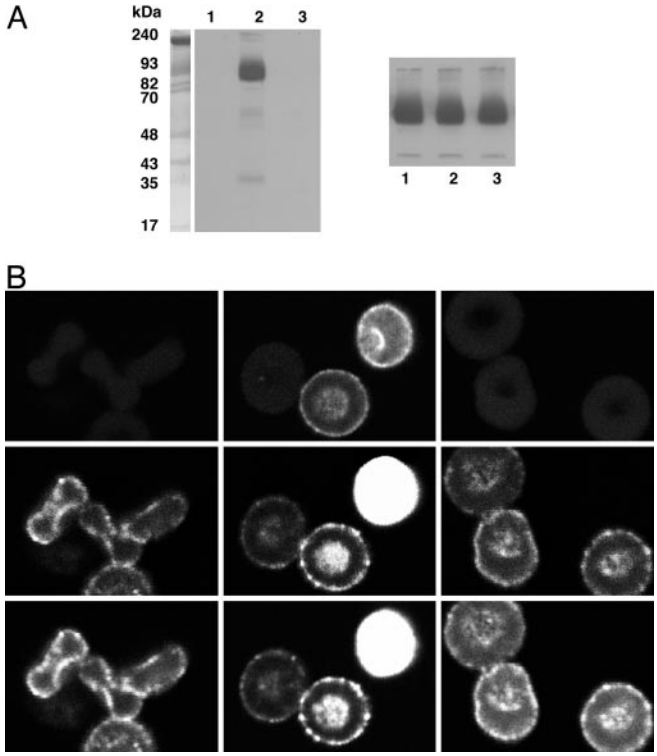

Fig. 5. Analysis of pervanadate-induced tyrosine phosphorylation of human RBCs by immunoblotting and confocal microscopy. Freshly drawn RBCs were stimulated with pervanadate, as described in *Materials and Methods*. (*A*) Membranes were prepared, separated by SDS/PAGE, and analyzed by immunoblotting first with a monoclonal anti-phosphotyrosine IgG (*Left*) and, after stripping, with a monoclonal anti-band 3 (*Right*). Lanes: 1, untreated control; 2, RBCs treated with pervanadate (see *Materials and Methods*); 3, RBCs treated first with staurosporine to block tyrosine phosphorylation and then with pervanadate as in lane 2. (*Left*) A Coomassie blue-stained lane of ghosts is aligned next to the blot. (*B*) RBCs stimulated with pervanadate as in *A* were fixed and triply stained with anti-phosphotyrosine (*Top*), anti-GAPDH (*Middle*), and anti-LDH (*Bottom*) before imaging by confocal microscopy. Micrographs of untreated control cells (*Left*), pervanadate-treated cells (*Center*), and staurosporine plus pervanadate-treated cells (*Right*) are shown. Note that the magnitude of enzyme displacement is proportional to the magnitude of tyrosine phosphorylation (see also Fig. 8).

near the NH_2 terminus of band 3 *in vivo*. Furthermore, the data clearly demonstrate that the association of the enzyme complex with the membrane is regulated by the oxygenation state of the cell.

A third line of evidence for assembly of a GE complex around cdb3 can be gleaned from studies of the effect of band 3 tyrosine phosphorylation on enzyme location. Thus, previous investigations of RBC tyrosine phosphorylation have shown that the vast majority of phosphotyrosine present in intact RBCs treated with pervanadate resides on band 3 (29) and that two of the most prominent tyrosine phosphorylation sites lie within the GE binding site at the NH_2 terminus of band 3 (30). Subsequent analysis has further demonstrated that this phosphorylation inhibits enzyme binding to cdb3 *in vitro* (31). To learn whether the same regulatory mechanism might be operative *in vivo*, we have induced band 3 tyrosine phosphorylation in intact cells and have examined the impact of this stimulation on GE distribution. As shown in the anti-phosphotyrosine immunoblots of Fig. 5*A*, treatment of RBCs with pervanadate induces a dramatic increase in tyrosine phosphorylation of band 3 but not of any other major membrane proteins. More importantly, as seen in Fig. 5*B*, RBCs that display an increase in tyrosine phosphorylation also show displacement of their GEs from the membrane. Furthermore, both pervanadate-induced tyrosine phosphorylation of band 3 (Fig. 5*A*) and GE displacement (Fig. 5*B*) are blocked by staurosporine, an inhibitor of band 3 tyrosine phosphorylation. We, therefore, conclude that band 3 phosphorylation leads to enzyme displacement *in vivo*, probably as a consequence of perturbation of the GE binding site at the NH_2 terminus of band 3. Because LDH and PK are also displaced by the same band 3 phosphorylation, we conclude that their association with the membrane depends on the integrity of the interaction of GAPDH, aldolase, and PFK with band 3. This property, which also mimics the other perturbations of band 3 described above, argues strongly that the GEs do not separately form autonomous associations with the membrane but rather interact in a macromolecular assembly where the fate of one enzyme is shared by all.

Epitope Occlusion Suggests That the Glycolytic Enzyme Complex Limits Ab Accessibility to Individual Enzymes. It has not escaped our notice that the fluorescence intensity of cells in which GEs have been displaced from the membrane is invariably greater than the fluorescence intensity of RBCs in which GEs remain membrane bound (Figs. 3–5). We interpret this observation to suggest that fewer of the enzymes' antigenic epitopes are accessible in the tightly assembled membrane complexes than in the freely diffusing enzymes. However, to place this observation on a more quantitative

foundation, we have fixed and stained oxygenated and deoxygenated cells and measured the average cell fluorescence by flow cytometry (data not shown). Except in the case of aldolase, the average fluorescence intensity of enzyme staining in oxygenated RBCs was less than one half of the intensity in their deoxygenated counterparts, suggesting that the epitopes are indeed more accessible after GE displacement from the membrane.

Discussion

We have provided several lines of evidence that GAPDH, aldolase, PFK, PK and LDH are not only membrane-associated in oxygenated RBCs but also assembled into a common GE complex organized around cdb3. First, a mAb that recognizes only residues 1–12 of band 3 was found to displace all of the GEs from membranes of cells into which the mAb was resealed. Although it might be argued that displacement of aldolase, GAPDH, and PFK is a trivial consequence of competition between the enzymes and the mAb for a common site on band 3, the same cannot be claimed for PK or LDH. Thus, neither PK nor LDH was found in three independent assays to bind band 3, suggesting that their displacement by anti-band 3 mAb occurs either because of their proximity to band 3 or because their binding site on the membrane requires the concurrent assembly of other GEs on band 3. This contention is further supported by observations that generation of deoxyHb or activation of tyrosine phosphorylation also displaces LDH and PK from the membrane, even though deoxyHb binding and tyrosine phosphorylation are thought to occur exclusively on cdb3. This selectivity for band 3 is especially demonstrable in the case of RBC tyrosine phosphorylation, where anti-phosphotyrosine immunoblots (Fig. 5) reveal no phosphorylation of any polypeptide other than band 3 present in sufficient quantities to bind LDH and PK. Thus, although no complex of GEs has ever been isolated from erythrocytes, the concerted release of multiple GEs by band 3 phosphorylation is most easily explained if all of the enzymes exist in a single complex nucleated around band 3, such that the stability of the entire complex depends on the association of band 3 with GAPDH, aldolase, and/or PFK. The inaccessibility of enzyme epitopes when present in the membrane-associated state is obviously consistent with this hypothesis.

What might constitute the function of a GE complex at the inner surface of the RBC membrane? The most frequently offered explanation for assembly of any enzyme complex argues that the efficiency of a pathway should increase with the channeling of substrates between sequential enzymes in a pathway (32). In this regard, it should be noted that there is mounting evidence that the glycolytic machinery in the human RBC can compartmentalize ATP, allowing its direct consumption by ion pumps without release into the cytoplasm (22–24). The enzyme complex identified in this study could obviously provide such a compartment. Furthermore, along somewhat different lines, it is also conceivable that collection of the enzymes into a single complex whose assembly is controlled by modulation of a single site (i.e., the NH_2 terminus of band 3) allows for more efficient regulation of a pathway. Thus, phosphorylation of band 3 or deoxygenation of Hb can lead to release of all enzymes from the membrane, even though only a single site on band 3 is modified. In this regard, it is interesting to note that red cell deoxygenation and tyrosine phosphorylation have been reported to accelerate glycolysis (33, 34).

Although already inferred in the data, one observation that is important to many aspects of red cell biology is worthy of special mention. That is, it can now be claimed with some confidence that the NH_2 terminus of band 3 indeed constitutes a binding site for GAPDH, aldolase, PFK, and deoxyHb in intact RBCs. If not, the effects of anti-band 3 mAb, Hb deoxygenation, and band 3 tyrosine phosphorylation on enzyme binding would be difficult to explain. We feel this conclusion deserves special note because it immediately suggests several testable hypotheses to account for the multiple changes in membrane properties associated changes in RBC oxygenation (35).

Given that the human RBC is often used as a model of more complex mammalian cells, the question finally arises whether similar GE complexes might exist in other cells. Although a definitive answer to this question cannot be offered, it is interesting to note that GEs have been found to associate with actin, tubulin, and troponin C (ref. 36 and unpublished observations), all of which contain the sequence AAYAA, where A is an acidic amino acid (i.e., glutamic acid or aspartic acid) and Y represents tyrosine. Because this same sequence constitutes the enzyme binding site at the NH_2 terminus of band 3 (see above), we suggest that a search for similar GE complexes in other cells is a justifiable endeavor.

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