The interaction of muscle phosphofructokinase with human erythrocyte ghost membranes was investigated. Scatchard analysis of the binding reveals only a single class of binding sites numbering approximately 4 x 10^4 sites/ghost with an association constant of 2 x 10^5 M^-1. Maximum binding is observed below pH 6.8 and is complete within 30 min at 4°C, 24°C, and 37°C; binding is inhibited by high ionic strength. Studies on the effect of ligands on the binding show that fructose-1,6-P2, Mg2+, and ATP favor binding, while ADP and 2,3-P2-glycerate do not.

The nature of the phosphofructokinase binding site on the membrane was investigated using membranes which had been washed with saline, EDTA, and NaOH to remove various peripheral membrane proteins. The results show that the removal of peripheral proteins from the membrane does not diminish the binding capacity of the membranes, suggesting that an integral membrane protein is the binding site.

Phosphofructokinase binding is inhibited by aldolase and glyceraldehyde-3-P dehydrogenase, which have been reported to bind to Band 3 protein (Kant, J. A., and Steck, T. L. (1973) J. Biol. Chem. 248, 8457-8464; Yu J., and Steck, T. L. (1975) J. Biol. Chem. 250, 8176-8184; Strapazon, E., and Steck, T. L. (1976) Biochemistry 15, 1421-1424; Strapazon, E., and Steck, T. L. (1977) Biochemistry 16, 2966-2971). Furthermore, both aldolase and glyceraldehyde-3-P dehydrogenase also mediate the dissociation of phosphofructokinase from these membranes; the amount of phosphofructokinase released is proportional to the amount of aldolase bound. Neither spectrin (0.1 mg/ml) nor hemoglobin (0.1 to 50 mg/ml) influences the binding of phosphofructokinase. These results demonstrate that phosphofructokinase binds to a specific site, probably Band 3 protein, on the inner surface of human erythrocyte membranes. A model which explains the relationship of the binding sites of these enzymes is presented.

Mammalian phosphofructokinase has been thought to reside exclusively within the cytoplasm of the cell because the enzyme is present in high speed supernatant fractions of tissue homogenates. However, we have demonstrated that phosphofructokinase binds to the inner surface of the human erythrocyte membrane (1). More importantly, the bound enzyme completely loses its allosteric characteristics, including a loss of inhibition by ATP, 2,3-P2-glycerate, and citrate (1).

Several other glycolytic enzymes, which were previously considered to exist only within the soluble fraction of the cell, have now been shown to bind tightly to cellular membranes. Glyceraldehyde-3-P dehydrogenase (2-9), fructose-1,6-bisphosphate aldolase (4-11), and phosphoglycerate kinase (12) have been shown to be bound to the human erythrocyte membrane. The binding of these enzymes is tight, but reversible, sensitive to high ionic strength, and pH-dependent. Certain physiologically significant metabolites influence the binding of these enzymes to the membrane. Both aldolase and glyceraldehyde-3-P dehydrogenase were shown to bind specifically to Band 3 protein (2, 3, 10, 11), the predominant integral polypeptide of the human erythrocyte membrane.

Using fluorescence quenching techniques, hemoglobin has also been found to be reversibly bound to the inner surface of the red cell membrane by an electrostatic interaction (13, 14). Competition studies have revealed that hemoglobin and glyceraldehyde-3-P dehydrogenase, but not aldolase, compete for binding sites, suggesting that Band 3 protein may be the binding site for hemoglobin.

In order to characterize the membrane-binding of phosphofructokinase, we have investigated the influence of ionic strength, pH, allosteric effectors, and other membrane-bound proteins upon the binding of phosphofructokinase to erythrocyte ghosts. Studies involving the competition for membrane binding of phosphofructokinase with aldolase, glyceraldehyde-3-P dehydrogenase, hemoglobin, and spectrin will be presented in this communication.

**MATERIALS AND METHODS**

Rabbit muscle phosphofructokinase was prepared according to the modified (15) procedure of Ling et al. (16). All other enzymes were purchased from Boehringer Mannheim. All chemicals and solvents were reagent grade and obtained from commercial sources.

**Polyacrylamide Gel Electrophoresis in SDS**—Electrophoresis was performed using 10% acrylamide gel containing 0.1% SDS according to the procedure of Laemmli (17). A protein solution was incubated with 1.0% SDS, 7.5% sucrose, 10 mM Tris/Cl (pH 8.0), 1.0 mM EDTA, 40 mM dithiothreitol and 10 μg/ml of pyronin Y at 37°C for 30 min. A sample (5 to 50 μg of protein) was layered on the gel and a current of 5 mA/gel was applied for 1.5 h. The gel was stained with Coomassie blue and destained in methanol/acetic acid. The destained gels were scanned at 600 nm using a Gifford spectrophotometer equipped with a linear transport device. The membrane polypeptides were enumerated by their characteristic mobilities according to Fairbanks et al. (18) and Steck (19).

**Preparation of Membranes**—Human erythrocyte membranes were prepared from freshly drawn or outdated blood obtained from local blood banks. Standard hemoglobin-free unsealed ghosts were prepared by the method of Fairbanks et al. (18). Saline (0.9% NaCl solution)-washed ghosts were obtained by washing the standard ghosts three times with 150 mM NaCl, 5 mM sodium phosphate, pH
8.0, followed by three washes with 5 mM sodium phosphate at pH 7 (2). EDTA-treated ghosts were prepared by incubating saline-washed ghosts in 0.1 mM EDTA, pH 6.0, at 37°C for 15 min (16). NaOH-treated ghosts were prepared by mixing saline-washed ghosts with 10 volumes of ice-cold 0.1 N NaOH and centrifuging (20).

Preparation of Hemoglobin—Hemoglobin was prepared according to the procedure of Marchesi (21). Saline-washed ghosts (10 mg/ml) were suspended in 10 volumes of 0.1 mM EDTA, pH 8.0, and incubated at 37°C for 15 min. After centrifugation at 100,000 χ g for 90 min, the resulting supernatant was concentrated using a Diaflo PM-30 membrane to a final concentration of 3 to 4 mg/ml. The pellet, which consisted of spectrin-depleted ghosts, was used in spectrin-binding studies. The preparations of spectrin were homogeneous and greater than 90% of the spectrin was removed in the spectrin-depleted ghosts as judged by SDSPAGE.

Preparation of Band 3 Protein—NaOH-washed ghosts were suspended in an equal volume of 3% Triton X-100 in 5 mM phosphate buffer at pH 7.5. To this suspension, 4 mg/ml of spectrin (0.4 units), 4 mM (NH4)2SO4, aldolase (0.4 units), triose-P isomerase (2.4 units), and α-glycerophosphate dehydrogenase (0.4 units). The reaction was initiated with the addition of phosphofructokinase, and the increase in absorbance at 340 nm was measured at 25°C. The specific activity of phosphofructokinase was 130 units/mg, as judged by SDS-PAGE.

Characterization of Phosphofructokinase Binding—In our previous studies, the protein was dialyzed overnight against 5 mM phosphate buffer, pH 7.0.

RESULTS

A few preliminary experiments have shown that the binding characteristics including the association constant (Kd) and the number of binding sites of rabbit muscle phosphofructokinase and human erythrocyte phosphofructokinase are similar. Since the muscle enzyme is more readily available than erythrocyte phosphofructokinase, the former was used in all our current studies.

Characterization of Phosphofructokinase Binding—In order to determine the dissociation constant and the number of binding sites on ghost membranes, saline-washed ghosts were incubated with varying amounts of phosphofructokinase. After centrifugation, both the pelleted membrane fraction and the supernatant solution were assayed for phosphofructokinase activity. The Scatchard plot (Fig. 1) indicates the presence of only one class of binding sites and also the nonlinearity may suggest a positive cooperativity. From the plot, an apparent association constant is estimated as 2 ± 0.2 x 10^-5 M^-1, and the number of binding sites/ghost is 4 ± 0.2 x 10^6 (average of eight experiments). This association constant for phospho-

![Fig. 1](https://example.com/fig1.png)
fructokinase is comparable to that of glyceraldehyde-3-P dehydrogenase (1 × 10⁻⁷ M⁻¹) (2) and aldolase (2.7 × 10⁻⁹ M⁻¹) (11). As discussed later, we tested the aldolase and glyceraldehyde-3-P dehydrogenase binding to our preparations of ghosts under the same conditions employed for phosphofructokinase, and our results show that the $K_A$ values for aldolase and glyceraldehyde-3-P dehydrogenase are 3 × 10⁻⁷ M⁻¹ and 2 × 10⁻⁹ M⁻¹, respectively. Thus, the $K_A$ for phosphofructokinase is similar to those for aldolase and glyceraldehyde-3-P dehydrogenase. The binding sites/ghost are reported to be 1.2 × 10⁶ (11) for aldolase and 1.3 to 1.7 (3, 8) for glyceraldehyde-3-P dehydrogenase, which are approximately 3 times more than that for phosphofructokinase.

**Rate of Binding and the Effect of Temperature**—The time course of phosphofructokinase binding to saline-washed ghosts is shown in Fig. 2. The results show that the binding is complete within 30 min at 5°C. The rates of binding are essentially the same at 24° and 37°C as at 5°C.

The Effect of pH—The effect of pH on the binding of phosphofructokinase to saline-washed ghosts was investigated. The results, as depicted in Fig. 3, show that maximum binding is obtained below pH 6.8, and the apparent $pK$ for half-maximum binding is approximately 7.1. The effect of pH on the release of bound phosphofructokinase from ghosts shows (data not shown) that the enzyme is not released at any pH below 6.6 and that at pH 7, approximately 50% of the bound enzyme dissociates from the membrane. Above pH 7.5, nearly all phosphofructokinase is released from the membrane. Thus, the association of phosphofructokinase with the ghost membrane is pH-dependent and completely reversible.

Effect of KCl—The association of phosphofructokinase with ghost membranes is inhibited in the presence of KCl at pH 7.5 (Fig. 4). Approximately 50% inhibition of the binding occurs at 35 mM KCl, and at 100 mM KCl only 10% of phosphofructokinase is bound to the membrane. At pH 6.5 and 7, this inhibition of binding is decreased, but since the enzyme is more unstable at lower pH values (resulting in poor recovery of the enzymatic activity), it is difficult to make any quantitative measurements.

Effect of Various Ligands on Dissociation of Bound Phosphofructokinase—The effect of physiological concentrations of various effectors of phosphofructokinase upon the dissociation of bound phosphofructokinase from ghost membranes was investigated. The results (Table I) show that under the conditions chosen, the control membranes (with no ligand present) release 96% of the bound enzyme. ATP, ITP, glucose-6-P, pyruvate, fructose-6-P, and MgSO₄ significantly protect against the dissociation of phosphofructokinase from the membrane and favor the binding of the enzyme. Binding studies (results not shown) in the presence of these ligands also confirm these results. For example, fructose-1,6-P₂, MgSO₄, and MgCl₂ significantly enhance the binding, while (27) of various effectors of phosphofructokinase upon the dissociation of bound phosphofructokinase from ghost membranes was investigated. The results (Table I) show that under the conditions chosen, the control membranes (with no ligand present) release 96% of the bound enzyme. ATP, ITP, glucose-6-P, pyruvate, fructose-6-P, and MgSO₄ significantly protect against the dissociation of phosphofructokinase from the membrane and favor the binding of the enzyme. Binding studies (results not shown) in the presence of these ligands also confirm these results. For example, fructose-1,6-P₂, MgSO₄, and MgCl₂ significantly enhance the binding, while...
Phosphofructokinase (130 μg) was allowed to bind to saline-washed ghosts (330 μg) in 5 mM potassium phosphate, pH 7.0, 1.5 mM ATP, and 1.5 mM MgSO₄. After 1-h incubation at 4°C, the mixture was centrifuged and the pellet containing the enzyme was incubated with the indicated ligands at pH 7.5. After incubation at 4°C for 1 h, the mixtures were centrifuged and phosphofructokinase activity in the supernatant and the pellet was determined.

The Stoichiometry of the Binding of Phosphofructokinase to Various Membrane Preparations—In order to examine the possible stoichiometric interaction between bound phosphofructokinase and Band 3 protein in the gel, the association constant (ZL) and the number of binding sites/ghost prepared from either fresh or aged blood (Table II). Similar results were obtained with EDTA-washed ghosts.

**Table I**
The effect of various ligands on the dissociation of phosphofructokinase from ghost membranes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Phosphofructokinase</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suspension</td>
<td>Supernatant</td>
<td>% released</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>units</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7.0</td>
<td>6.7</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>ATP (1.5)</td>
<td>7.4</td>
<td>5.6</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>ADP (0.2)</td>
<td>7.5</td>
<td>6.7</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>AMP (0.008)</td>
<td>7.3</td>
<td>6.1</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ (2.5)</td>
<td>8.1</td>
<td>4.9</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P (0.039)</td>
<td>7.1</td>
<td>5.1</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Fructose-6-P (0.016)</td>
<td>7.3</td>
<td>6.9</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-P (0.007)</td>
<td>7.3</td>
<td>4.7</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>2,3-P₂-glycerate (6.0)</td>
<td>6.5</td>
<td>5.7</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Pyruvate (0.085)</td>
<td>6.6</td>
<td>5.1</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>TTP (2.0)</td>
<td>7.8</td>
<td>5.7</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

2,3-P₂-glycerate and ADP inhibit the binding. In the presence of 4 to 8 mM MgSO₄ or MgCl₂ and 33 mM ATP, over 70% of phosphofructokinase is bound to saline-washed ghosts.

**Phosphofructokinase Binding to Membrane Ghosts Prepared from Fresh Blood**—Since the results reported above were obtained using ghosts prepared from blood stored for 3 weeks, the binding of phosphofructokinase to ghosts prepared from freshly drawn blood was examined. The binding constants and the number of binding sites/ghost prepared from fresh blood are identical with those of ghosts prepared from 3-week-old blood (Table II). The membrane prepared from blood which had been stored over 4 weeks, however, contains significantly fewer binding sites than those of fresh blood (Table II).

**Phosphofructokinase Binding to Various Ghost Preparations**—In order to characterize further the binding of phosphofructokinase to ghost membranes, various membrane preparations differing in protein composition were examined: (a) saline-washed ghosts which lack glyceraldehyde-3-P dehydrogenase (Band 6) (2); (b) EDTA-treated membranes which lack Bands 1, 2, 5, and 6 (18); and (c) NaOH-washed ghosts lacking all peripheral proteins while retaining Band 3 protein (20). Excess phosphofructokinase was incubated with saline-washed ghosts as well as NaOH-treated membranes and the amount of bound phosphofructokinase was determined. The results (Fig. 5) indicate that the amount of phosphofructokinase bound to the NaOH-washed membranes which contain predominantly Band 3 protein is the same as the amount bound to saline-washed ghosts when corrected for the content of Band 3 protein in this membrane. Furthermore, the association constant of the enzyme for this membrane is the same as that of saline-washed ghosts prepared from either fresh or aged blood (Table II). Similar results were obtained with EDTA-washed ghosts.

**FIG. 5.** Phosphofructokinase binding to different preparations of ghosts. Phosphofructokinase (PFK) (60 to 360 μg) was mixed with either saline-washed ghosts (●) (310 μg) of NaOH-treated ghosts (○) (160 μg) in 1 ml of 5 mM potassium phosphate, pH 7.0, 2 mM ATP, and 2 mM MgCl₂ at 4°C for 60 min. The mixture was centrifuged and assayed for phosphofructokinase activity as described under "Materials and Methods." The concentration of Band 3 protein in these membrane preparations was determined by gel electrophoresis as described under "Materials and Methods."

**Table II**
The association constants and the number of binding sites of membranes prepared from various blood sources

<table>
<thead>
<tr>
<th>Ghosts</th>
<th>Kₐ (×10⁻⁹ M⁻¹)</th>
<th>Number of binding sites/ghost</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh blood</td>
<td>1.7</td>
<td>4.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>3-week-old blood</td>
<td>1.8</td>
<td>4.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>NaOH-treated</td>
<td>1.7</td>
<td>4.2 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

**Table III**
Molar ratios of phosphofructokinase and Band 3 dimer protein in the phosphofructokinase-Band 3 complex

<table>
<thead>
<tr>
<th>Ghosts</th>
<th>Concentration</th>
<th>Molar ratio in the complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Band 3</td>
<td>Photophructokinase</td>
</tr>
<tr>
<td>EDTA-treated</td>
<td>5.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Saline-washed</td>
<td>6.2</td>
<td>10.6</td>
</tr>
<tr>
<td>NaOH-treated</td>
<td>7.3</td>
<td>10.7</td>
</tr>
<tr>
<td>Average</td>
<td>6.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

**Note:** The Stoichiometry of the Binding of Phosphofructokinase to Various Membrane Preparations—In order to examine the possible stoichiometric interaction between bound phosphofructokinase and Band 3 protein in the gel, the association constant (ZL) and the number of binding sites/ghost prepared from either fresh or aged blood (Table II). Similar results were obtained with EDTA-washed ghosts.
with our preparation, it is possible that the stoichiometry is 2 mol of enzyme/mol of Band 3 dimer.

The Effect of Aldolase and Glycerdehyde-3-P Dehydrogenase on the Binding of Phosphofructokinase—Since aldolase and glycerdehyde-3-P dehydrogenase have been reported to bind to Band 3 protein (2, 3, 10, 11), it is of interest to determine what effect those enzymes may have on the binding of phosphofructokinase to erythrocyte membranes. Aldolase or glycerdehyde-3-P dehydrogenase was first bound maximally to saline-washed ghosts, and then the ghosts were allowed to react with phosphofructokinase. As shown in Fig. 6, A and B, aldolase or glycerdehyde-3-P dehydrogenase inhibit the binding of phosphofructokinase. The maximum binding of phosphofructokinase was decreased by approximately 70% in the presence of 60 μg of aldolase or 140 μg of glycerdehyde-3-P dehydrogenase. The double reciprocal plot (not shown) indicates that inhibition is noncompetitive; however, the nature of the inhibition cannot be determined since the lines are nonlinear and the points are scattered, especially at high phosphofructokinase concentrations.

The effect of both glycerdehyde-3-P dehydrogenase and aldolase on the binding of phosphofructokinase to saline-washed ghosts is shown in Fig. 7. The results show a significant inhibition of phosphofructokinase binding by bound glycerdehyde-3-P dehydrogenase and aldolase; however, the effect is not additive if compared to either aldolase or glycerdehyde-3-P dehydrogenase alone. These results may be due to the competition of glycerdehyde-3-P dehydrogenase and aldolase with phosphofructokinase for membrane binding sites. Fig. 8 shows the effect of glycerdehyde-3-P dehydrogenase on the binding of aldolase to saline-washed ghosts. The Scatchard plots (Fig. 8, inset) of the effect of varying concentrations of glycerdehyde-3-P dehydrogenase on the binding of aldolase to the membrane show that the slopes of the lines remain constant, indicating that the binding constant (Kd) of aldolase is unaltered by the bound glycerdehyde-3-P dehydrogenase. The number of binding sites for aldolase (intercepts on the X axis), however, is decreased from 1 to 0.2 with increasing concentrations of bound glycerdehyde-3-P dehydrogenase.

Fig. 6. The effect of aldolase or glycerdehyde-3-P dehydrogenase on the binding of phosphofructokinase (PFK) to unsealed ghosts. A, saline-washed ghosts were incubated with varying concentrations of aldolase (0 μg/ml (●), 20 μg/ml (△), 60 μg/ml (○)) in 5.5 ml of 5 mM potassium phosphate, pH 7.0, at 4°C for 1.5 h, and then centrifuged to isolate aldolase-bound ghosts. The pellet was suspended in 0.5 ml of the buffer. An aliquot (50 μl) of the suspended pellet was then incubated with 0.5 ml of varying concentrations of phosphofructokinase in the presence of 1 mM ATP, 1 mM MgCl2, and 1 mM dithiothreitol for 1 h at 4°C. The mixture was centrifuged and assayed for phosphofructokinase activity. Under these conditions, about 90% of aldolase was bound to the membrane. B, glycerdehyde-3-P dehydrogenase-bound ghosts were prepared as in A except for the enzyme (140 μg). Approximately 85% of glycerdehyde-3-P dehydrogenase was bound to the membrane. The binding of phosphofructokinase to this membrane was performed as in A.

Fig. 7. The effect of glycerdehyde-3-P dehydrogenase and aldolase on the binding of phosphofructokinase to unsealed ghosts. Saline-washed ghosts (700 μg) in 4.4 ml of 5 mM potassium phosphate, pH 7.0, containing 1 mM dithiothreitol, 2 mM ATP, and 2 mM MgCl2 were combined with aldolase (100 μg, (●)), glycerdehyde-3-P dehydrogenase (300 μg, (○)), or a mixture of both enzymes (△). A control (○) without these enzymes was also included. The mixtures were incubated, and the activity of membrane-bound phosphofructokinase was determined as described in the legend to Fig. 6.
Membrane-bound Phosphofructokinase

FIG. 8. The effect of glyceraldehyde-3-P dehydrogenase on the binding of aldolase to unsealed ghosts. Saline-washed ghosts (1.4 mg) were mixed with 1.2 mg (A), 0.6 mg (△), 0.3 mg (○), and 0 mg (O) of glyceraldehyde-3-P dehydrogenase in 4.4 ml of 5 mM potassium phosphate, pH 7.5. The mixture was incubated for 1.5 h at 4°C and centrifuged and the pellet was suspended in 0.4 ml of the buffer. The suspended pellet (50 μl) was then combined with varying concentrations of aldolase in 0.5 ml of the same buffer and incubated for 1.5 hr at 4°C. The membrane-bound aldolase was determined as described under "Materials and Methods."

The Effect of Glyceraldehyde 3-P Dehydrogenase or Aldolase on the Release of Phosphofructokinase Bound to Saline-washed Ghosts—Aldolase-mediated dissociation of phosphofructokinase from saline-washed ghosts is shown in Fig. 9. The amount of phosphofructokinase released is directly proportional to the amount of aldolase bound. The slopes of the linear portions of the curves indicate that approximately 1 mol of phosphofructokinase is released from the membrane when 1.9 mol of aldolase are bound to the membrane. These observations further support our evidence (Table III) that the stoichiometry of binding is 1 mol of phosphofructokinase (tetramer)/mol of Band 3 dimer. (The stoichiometry of the binding of aldolase has been reported to be 2 mol of enzyme/Band 3 dimer (11))

Glyceraldehyde-3-P dehydrogenase also displaces the bound phosphofructokinase from the membrane, but approximately 10 to 60 mol of glyceraldehyde-3-P dehydrogenase are required in order to displace 1 mol of phosphofructokinase.

TABLE IV
The effect of hemoglobin on the release of phosphofructokinase bound to saline-washed ghosts

<table>
<thead>
<tr>
<th>Hemoglobin added (mg/ml)</th>
<th>Hemoglobin bound</th>
<th>Phosphofructokinase released (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>0.9</td>
<td>0.2</td>
<td>26</td>
</tr>
<tr>
<td>2.7</td>
<td>0.6</td>
<td>24</td>
</tr>
<tr>
<td>5.4</td>
<td>1.5</td>
<td>24</td>
</tr>
<tr>
<td>9.9</td>
<td>1.5</td>
<td>25</td>
</tr>
<tr>
<td>25.2</td>
<td>6.9</td>
<td>25</td>
</tr>
<tr>
<td>50.5</td>
<td>7.8</td>
<td>24</td>
</tr>
</tbody>
</table>

The binding of phosphofructokinase to erythrocyte ghosts. Thus, after saline-washed ghosts were bound maximally with phosphofructokinase, varying concentrations (0 to 50 mg/ml) of hemoglobin were added and the amount of phosphofructokinase released was measured. As shown in Table IV, the binding of hemoglobin to the membrane did not displace bound phosphofructokinase above control values. Therefore, hemoglobin and phosphofructokinase appear to bind to different sites on the erythrocyte membrane.

The Effect of Spectrin on the Binding of Phosphofructokinase—Spectrin, a peripheral membrane protein, has recently been shown to bind to a 72,000-dalton fragment generated by α-chymotrypsin digestion of erythrocyte membrane ghosts (29, 30). Since the NaOH-washed ghost preparations may contain the spectrin-binding protein as well as Band 3...
We have shown previously that phosphofructokinase binds to the inner surface of the human erythrocyte membrane (1). Our current study suggests that the enzyme binds to a specific site on the membrane. A linear Scatchard plot resulting from the binding of phosphofructokinase to ghost membranes suggests the presence of only a single class of binding sites with an association constant of $1.7 \times 10^{10} \text{ M}^{-1}$ (Fig. 1). Phosphofructokinase binds to various membrane ghost preparations which have been treated with salt, EDTA, or NaOH to remove peripheral membrane proteins (Fig. 5). Furthermore, the amount of enzyme bound is directly proportional to the amount of Band 3 protein in these membranes (Table III). Competition studies show that both aldolase and glyceraldehyde-3-P dehydrogenase compete with phosphofructokinase (Figs. 6 and 7) as well as with each other (Fig. 8) for binding sites on the membrane. Since both aldolase and glyceraldehyde-3-P dehydrogenase have been reported to bind to Band 3 protein on the inner surface of the human erythrocyte (2, 3, 10, 11), our data suggest that phosphofructokinase is likewise binding to Band 3 protein. Furthermore, we show that both aldolase and glyceraldehyde-3-P dehydrogenase mediate the release of membrane-bound phosphofructokinase (Fig. 9). However, while the glyceraldehyde-3-P dehydrogenase-mediated release of phosphofructokinase is not stoichiometric, aldolase stoichiometrically displaces membrane-bound phosphofructokinase (Fig. 9). One mol of phosphofructokinase is released from the membrane when 2 mol of aldolase are bound. These results further suggest that phosphofructokinase and aldolase may bind to the same site or neighboring sites and that glyceraldehyde-3-P dehydrogenase binds to a more distant site. Thus, one may envision a series of overlapping binding sites, spatially arranged on the Band 3 protein, such that the phosphofructokinase binding site is closer to that of aldolase than to that of glyceraldehyde-3-P dehydrogenase.

Consistent with our proposed model of binding sites is the lack of competition of phosphofructokinase (Table IV) or aldolase (14) with hemoglobin for membrane binding sites. The addition of as much as 50 mg/ml of hemoglobin did not affect the release of membrane-bound phosphofructokinase (Table IV). Although this concentration of hemoglobin is somewhat lower than the physiological level of hemoglobin in red cells (340 mg/ml), the membrane binding sites are saturated with hemoglobin. Furthermore, the membrane binding constant for hemoglobin ($K_a = 10^{10} \text{ M}^{-1}$) (14) indicates a stronger membrane interaction relative to that of phosphofructokinase ($1.7 \times 10^{10} \text{ M}^{-1}$, Fig. 1). Thus, if the two proteins bound to the same site, the binding of hemoglobin would be greatly favored and, therefore, bound phosphofructokinase would be displaced. In addition, hemoglobin has been reported to compete with glyceraldehyde-3-P dehydrogenase, but not with aldolase, for binding sites on the erthrocyte membrane, presumably on Band 3 protein (14). Thus, although hemoglobin and glyceraldehyde-3-P dehydrogenase may be competing for the same or adjacent site(s), the binding site for hemoglobin appears to be different from that of phosphofructokinase and aldolase.

Also consistent with our proposed model are the competition studies between spectrin and phosphofructokinase (Fig. 10) for binding sites. We observed no competition between spectrin and phosphofructokinase for binding sites (Fig. 10), suggesting that spectrin and phosphofructokinase bind to different sites. This is further supported by the recent reports that spectrin binds to Band 2.1 protein (33, 34) rather than Band 3 protein.

We have no direct physical evidence for an interaction between Band 3 protein and phosphofructokinase; however, a complex between Band 3 protein and glyceraldehyde-3-P dehydrogenase (2, 3) or aldolase (10, 11) has been demonstrated in the presence of Triton X-100 using sucrose density gradient centrifugation (2, 3, 10, 11). Solubilized Band 3 protein was reported to form a complex with purified glyceraldehyde-3-P dehydrogenase, suggesting that spectrin binds to Band 2.1 protein (33, 34) rather than Band 3 protein. Furthermore, we show that both aldolase and glyceraldehyde-3-P dehydrogenase mediate the release of membrane-bound phosphofructokinase (Fig. 9). However, while the glyceraldehyde-3-P dehydrogenase-mediated release of phosphofructokinase is not stoichiometric, aldolase stoichiometrically displaces membrane-bound phosphofructokinase (Fig. 9). One mol of phosphofructokinase is released from the membrane when 2 mol of aldolase are bound. These results further suggest that phosphofructokinase and aldolase may bind to the same site or neighboring sites and that glyceraldehyde-3-P dehydrogenase binds to a more distant site. Thus, one may envision a series of overlapping binding sites, spatially arranged on the Band 3 protein, such that the phosphofructokinase binding site is closer to that of aldolase than to that of glyceraldehyde-3-P dehydrogenase.

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Discussion

We have shown previously that phosphofructokinase binds to the inner surface of the human erythrocyte membrane (1). Our current study suggests that the enzyme binds to a specific site on the membrane. A linear Scatchard plot resulting from the binding of phosphofructokinase to ghost membranes suggests the presence of only a single class of binding sites with an association constant of $1.7 \times 10^{10} \text{ M}^{-1}$ (Fig. 1). Phosphofructokinase binds to various membrane ghost preparations which have been treated with salt, EDTA, or NaOH to remove peripheral membrane proteins (Fig. 5). Furthermore, the amount of enzyme bound is directly proportional to the amount of Band 3 protein in these membranes (Table III). Competition studies show that both aldolase and glyceraldehyde-3-P dehydrogenase compete with phosphofructokinase (Figs. 6 and 7) as well as with each other (Fig. 8) for binding sites on the membrane. Since both aldolase and glyceraldehyde-3-P dehydrogenase have been reported to bind to Band 3 protein on the inner surface of the human erythrocyte (2, 3, 10, 11), our data suggest that phosphofructokinase is likewise binding to Band 3 protein. Furthermore, we show that both aldolase and glyceraldehyde-3-P dehydrogenase mediate the release of membrane-bound phosphofructokinase (Fig. 9). However, while the glyceraldehyde-3-P dehydrogenase-mediated release of phosphofructokinase is not stoichiometric, aldolase stoichiometrically displaces membrane-bound phosphofructokinase (Fig. 9). One mol of phosphofructokinase is released from the membrane when 2 mol of aldolase are bound. These results further suggest that phosphofructokinase and aldolase may bind to the same site or neighboring sites and that glyceraldehyde-3-P dehydrogenase binds to a more distant site. Thus, one may envision a series of overlapping binding sites, spatially arranged on the Band 3 protein, such that the phosphofructokinase binding site is closer to that of aldolase than to that of glyceraldehyde-3-P dehydrogenase.

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dehye-3-P dehydrogenase, while aldolase was first bound to the membrane and the aldolase-Band 3 complex was then extracted in Triton X-100. Both the aldolase-Band 3 complex and the glyceraldehyde-3-P dehydrogenase-Band 3 complex were found to be sensitive to high ionic strength and certain metabolites, thus indicating the specificity of the interaction. We attempted, although unsuccessfully, to demonstrate a putative phosphofructokinase-Band 3 protein complex using similar methods. One explanation for our inability to demonstrate the complex is the relatively weak binding of phosphofructokinase (Kᵢ = 1.7 × 10⁻³ M⁻¹, Fig. 1) as compared to that of glyceraldehyde-3-P dehydrogenase (Kᵢ = 1.0 M⁻¹) (10).

Another possible explanation is that the Band 3 protein may undergo a conformational change or dissociate into a form incapable of binding phosphofructokinase. The stoichiometry of the binding of phosphofructokinase appears to be 1 mol of enzyme (Mₑ = 320,000)/one dimer of Band 3 protein (Mₑ = 180,000) (Table III, Fig. 9). Therefore, the monomeric form of Band 3 protein probably cannot bind phosphofructokinase. In contrast, the stoichiometry of the binding of aldolase or glyceraldehyde-3-P dehydrogenase is 1 mol of enzyme/mol of Band 3 polypeptide (Mₑ = 90,000) or 2 mol one dimer of Band 3 protein (3, 11). Therefore, another approach is necessary to demonstrate the formation of a phosphofructokinase-Band 3 complex involving weak interactions.

Assuming that all three enzymes bind to Band 3 protein, the question may be raised as to whether there is sufficient Band 3 protein molecules in vitro to bind all three enzymes. It has been estimated that there are 1.2 × 10⁶ Band 3 polypeptides (or 5 × 10⁷ dimers)/ghost (19). Furthermore, Kant and Steck (2) have calculated that there are 1.4 to 2.4 × 10⁶ glyceraldehyde-3-P dehydrogenase molecules/erythrocyte, so that this enzyme could occupy maximally 1 to 24% of the binding sites. It can also be estimated from the total activities of aldolase (35) and phosphofructokinase (36) that there are 1 to 2 × 10⁶ molecules/erythrocyte and 1.5 to 3 × 10⁶ molecules/erythrocyte, respectively. This means that, at most, 10 to 26% and 3 to 8% of the binding sites could be bound with aldolase and phosphofructokinase molecules, respectively. Thus, there are more than adequate Band 3 protein molecules in vitro to provide the potential binding sites for these three enzymes.

It has been estimated from the dimensions of glyceraldehyde-3-P dehydrogenase that 2 × 10⁶ molecules bound at saturation would occupy an area of about 37 to 74 μm² or about 30 to 60% of the total area (132 μm²) of the inner membrane surface (37). Such a large fraction of surface covered by these enzyme molecules raises the possibility that when these enzymes compete for the binding site, steric hindrance, rather than the number of available sites, limits the binding of these proteins. This may explain the complex inhibition (uncompetitive or noncompetitive) patterns observed for the binding of phosphofructokinase by the other enzymes (Figs. 6 and 7), as well as the interference of glyceraldehyde-3-P dehydrogenase (Fig. 8) in the binding of aldolase to the membrane.

The binding of phosphofructokinase to the membrane is greatly influenced by various ligands. Since these ligands are the substrate, the products, and the allosteric effectors of the enzyme, conformational changes induced by these metabolites may determine the degree of the enzymes' interaction with the membrane site. Fructose-1,6-P₂ at low concentrations (7 μM) favors the association of phosphofructokinase with the membrane (Table I). It is surprising that fructose-6-P does not have any effect on the interaction while ATP, the other substrate, promotes the binding. MgSO₄ (2.5 mM) as well as MgCl₂ (not shown) greatly favor the binding of phosphofructokinase to the membrane. Thus, Mg²⁺ is probably responsible for this effect (Table I).

Phosphofructokinase, like the other enzymes, appears to interact ionically with the membrane protein, since high ionic strength dissociates the enzyme from the membrane. The mechanism by which phosphofructokinase and other enzymes bind membranes in vitro remains to be elucidated, since cytoplasmic ionic strength and certain metabolites promote dissociation in vitro (10, 12, 24). There are no known physiological requirements for the binding of any of these enzymes, with the possible exception of phosphofructokinase. Membrane-bound phosphofructokinase loses allosteric inhibition of ATP and 2,3-P₂-glycerate (1), which are known regulators of this enzyme in erythrocytes. The relationship of the binding of glycolytic enzymes to the erythrocyte membrane in vitro and the regulatory control of glycolysis in vitro has not been established. However, dissociation of the enzyme-membrane complex in vitro by physiological saline does not exclude the possible interaction between these glycolytic enzymes and the membrane in vivo. Currently, we are not aware of various factors which might promote this interaction in vivo such as the microenvironment around the enzymes including the ionic strength, pH, and the concentrations of proteins and ligands. For example, our dissociation experiments were performed in a volume at least 1 order of magnitude larger than that in the cytoplasm. Furthermore, aldolase and other glycolytic enzymes have been shown to bind to myosins at physiological pH and ionic strength only at relatively high concentrations of these proteins (approaching the physiological levels) (38).

Since the membrane contains specific binding sites for these enzyme(s) and 10 to 20% of phosphofructokinase is bound to the membrane when red cells are hemolyzed, the possibility that this interaction is of physiological significance is strengthened.

Several sequential glycolytic enzymes, including phosphofructokinase, aldolase, glyceraldehyde-3-P dehydrogenase, and 3-P glyceralate kinase, are now known to partition between the membrane and the cytoplasm. The partition is influenced by ionic strength, pH, various ligands and other enzymes. The binding of these sequential enzymes may provide an increased catalytic efficiency for the overall reaction, since the product of one enzyme is immediately available for the next enzyme without being released into the medium. In addition, membrane-bound phosphofructokinase is no longer susceptible to inhibition by the negative effectors such as ATP and 2,3-P₂-glycerate. Thus, the net result of these two factors, namely (a) increased catalytic efficiency and (b) loss of the allosteric inhibition of phosphofructokinase, would greatly enhance glycolysis within the cell. However, further experimentation is required to explore these possibilities and to define the physiological role of the association of these glycolytic enzymes.

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Membrane-bound Phosphofructokinase

The interaction of phosphofructokinase with erythrocyte membranes.
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