Changes in Allosteric Properties of Phosphofructokinase Bound to Erythrocyte Membranes*  

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SUMMARY  

Human and rabbit erythrocyte membranes prepared by hypotonic hemolysis contained 5 to 15% of the phosphofructokinase in the erythrocytes. The membrane-bound phosphofructokinase can be eluted by a saline wash. Human erythrocyte and rabbit muscle phosphofructokinase bind to the saline-washed membranes. This binding is specific for the inner surface of the membrane. The amount of phosphofructokinase bound is dependent on pH; at pH 7, 6 times more enzyme is bound than at pH 7.5. Unlike free phosphofructokinase, the membrane-bound phosphofructokinase is not inhibited by ATP or 2,3-diphosphoglycerate, and its fructose-6-P saturation curve is nonsigmoidal.

The glycolytic enzymes, aldolase (1-5) and glyceraldehyde-3-P dehydrogenase (5-7) have been found to be associated with erythrocyte membrane ghosts. All mammalian phosphofructokinases are soluble proteins, with no evidence of binding to any membrane thus far reported.

In this communication, we have investigated the binding of human erythrocyte and rabbit muscle phosphofructokinase to human erythrocyte membranes and some resulting changes in the allosteric properties of the enzyme.

MATERIALS AND METHODS  

Human erythrocyte phosphofructokinase was purified according to the procedure of Karadsheh et al. (8) and stored at −70°. Rabbit muscle phosphofructokinase was prepared with our modified procedure (9) of the method of Ling et al. (10).

Standard hemoglobin-free unsealed ghosts were prepared by the method of Fairbanks et al. (11) from freshly outdated human blood obtained from a blood bank then stored at 0° or in liquid N₂. Saline-washed ghosts devoid of phosphofructokinase were prepared by washing the standard ghosts with 150 mM NaCl, 5 mM potassium phosphate, pH 7.5, followed by three washings with 5 mM potassium phosphate, pH 7.5. Resealed ghosts were prepared in 1 mM MgSO₄, 5 mM potassium phosphate, pH 8, according to the procedure of Kant and Steck (12). Ghosts containing bound phosphofructokinase were prepared by incubating approximately 100 units (1.2 mg) of phosphofructokinase with the saline-washed ghosts containing 4.7 mg of protein in 5 mM potassium phosphate, pH 7.5, for 1 h at 0°. The membranes were centrifuged, washed three times with 5 ml each of 5 mM potassium phosphate, pH 7.5, and suspended in 0.8 ml of the same buffer. Under these conditions, the amount of phosphofructokinase bound was usually 10 units.

Phosphofructokinase activity was determined two ways. Assay A, for optimum activity, contained in a final volume of 1 ml: 50 mM Tris/Cl (pH 8), 1 mM EDTA, 6 mM MgCl₂, 2.5 mM diethiothreitol, 0.16 mM NADH, 1 mM ATP, 1 mM fructose-6-P, aldolase (0.4 unit), triose-P isomerase (2.4 units), and α-glycerol-P dehydrogenase (0.4 unit). The reaction was initiated with phosphofructokinase and the decrease in absorbance at 540 nm was measured at 95° with a Gilford spectrophotometer. Assay B, for studying allosteric effects, contained in a final volume of 1 ml: 50 mM Tris/Cl (pH 7.5), 1 mM NH₄Cl, 9.5 mM diethiothreitol, 0.16 mM NADH, indicated amounts of ATP, fructose-6-P, MgCl₂, and the same number of units of desalted coupling enzymes used for Assay A with the reaction initiated and monitored as above.

Glyceraldehyde-3-P dehydrogenase was assayed according to the procedure of Strapazon and Steck (13) and aldolase activity was determined according to the procedure of Stock and Kant (14). Protein was determined by the Lowry phenol method (15).

RESULTS  

Binding of Phosphofructokinase to Membrane Ghost  

The glyceraldehyde-3-P dehydrogenase, aldolase, and phosphofructokinase content of standard hemoglobin-free, unsealed ghosts prepared from human and rabbit erythrocytes is presented in Table I. Human ghost preparations contain approximately 5 and 15% of total phosphofructokinase and aldolase, respectively, in the erythrocytes. On the other hand, rabbit ghosts contain 13% and less than 10% of phosphofructokinase and aldolase, respectively.

The binding of human erythrocyte phosphofructokinase to depleted erythrocyte membrane ghosts appears to follow a simple saturation curve (Fig. 1), but the gradual increase in binding above 5 units of phosphofructokinase may indicate existence of another class of binding sites. In separate studies (not shown), it was found that the binding of phosphofructokinase to the membrane reached equilibrium within 45 min at 0°. The amount of phosphofructokinase bound is estimated as approximately 10 units/mg of membrane protein under these conditions.

Resealed ghosts which expose the outer surface of membrane to the external medium bound less than one-fourth of the amount of phosphofructokinase bound to unsealed ghosts (Fig. 1). Microscopic examination showed that the resealed ghosts contained some unsealed ghosts which may account for part of this small amount of binding. The apparent nonlinear shape of this curve is likely due to random error in the measurement of small amounts of bound phosphofructokinase. It is also possible that this binding may be phosphofructokinase binding to the second class of sites. These results

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show that most of the phosphofructokinase binding is specific to the inner surface of the membrane which is exposed to the cytoplasm.

A change in pH has a profound effect on the binding of phosphofructokinase to the membrane. 6 times more phosphofructokinase is bound at pH 7.0 than at pH 7.5, and less than 1/10 as much phosphofructokinase is bound at pH 8 compared to pH 7.

**Kinetic Characteristics of Membrane-bound Phosphofructokinase**

**ATP Inhibition**—Free phosphofructokinase from muscle or erythrocyte is inhibited strongly at high concentrations of ATP, a common property of all mammalian phosphofructokinases. However, the membrane-bound phosphofructokinase (erythrocyte as well as muscle) completely loses this sensitivity to ATP inhibition (Fig. 2). Since ATP inhibition is known to be greater at lower pH (16), the inhibition was examined also at pH 7. The activity curves at pH 7 (not shown) look similar to those shown in Fig. 2, with no inhibition of the membrane-bound phosphofructokinase.

At high concentrations of ATP, a plot of the activity of free phosphofructokinase as a function of fructose-6-P concentration is sigmoidal in shape. However, a similar plot for membrane-bound phosphofructokinase is nonsigmoidal (Fig. 3).

The behavior of phosphofructokinase first bound to the membranes then dissociated and separated from the membrane is the same as the free enzyme. Thus, any change in phosphofructokinase conformation involved in binding to the membrane is reversible upon its dissociation from the membranes.

**2,3-Diphosphoglycerate Inhibition**—Erythrocyte phosphofructokinase is known to be inhibited by high concentration of 2,3-diphosphoglycerate synergistically with ATP. It is of interest then to compare inhibition of the free and the bound phosphofructokinase by this negative effector. The results depicted in Fig. 4 show that the bound enzyme looses this inhibition also.

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Fig. 1. Binding of human erythrocyte phosphofructokinase (PFK) to unsealed and resealed membranes. Varying concentrations of human erythrocyte phosphofructokinase were incubated with the depleted unsealed ghosts (○) or Mg++-resealed ghosts (○) (0.63 mg of protein) in 1 ml of 5 mM phosphate, pH 7.5, for 1 h at 0° and centrifuged, and the supernatant solution as well as pellet was assayed for phosphofructokinase.

Fig. 2. Effect of ATP concentrations on free and bound phosphofructokinase (PFK) activities versus ATP. The membrane-bound human erythrocyte (A) and rabbit muscle (B) phosphofructokinases were prepared as described under "Materials and Methods." Assay B except for the indicated concentration of ATP and 0.2 mM fructose-6-P were used. ○—○, free; •—•, membrane-bound phosphofructokinase.

Fig. 3. Fructose-6-P saturation curves for bound and free phosphofructokinase (PFK). The membrane-bound human erythrocyte phosphofructokinase (○) and free phosphofructokinase (●) were assayed in Assay B except 1 mM ATP and 3 mM MgCl₂ were used.
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![Graph](image)

**Fig. 4.** Effect of 2,3-diphosphoglycerate on free and bound phosphofructokinase activities. The membrane-bound human erythrocyte phosphofructokinase (□) was assayed in Assay B except 1 mM ATP and 3 mM MgCl₂ were used. The free enzyme (O) was assayed in the same assay mixture as that for the membrane enzyme except 0.13 mM ATP and 0.38 mM MgCl₂, because the free enzyme is extremely sensitive to ATP inhibition.

**DISCUSSION**

Our present results show that phosphofructokinase bound to red cell membranes loses its sensitivity to ATP and 2,3-diphosphoglycerate inhibition. The degree of binding to the membrane may play an important role in the regulation of this enzyme and glycolysis in vivo. The rate of glycolysis in human red cells is approximately 0.03 μmol of glucose utilized/min/ml of cells (17). Phosphofructokinase, the most important control enzyme of this pathway, is present at 2 to 4.5 units/ml. Thus, the enzyme should be operating at about 1% of the maximum catalytic capacity. If the known concentrations of inhibitors such as ATP and 2,3-diphosphoglycerate present in the cells are taken into consideration, the actual activity of phosphofructokinase is probably less than 0.1% of the maximum rate, that is 0.002 to 0.0045 unit/ml. Rose and Warms (18) have shown that glucose-1,6-P₂ and mannose-1,6-P₂ are present in high enough concentration in the cells to raise the phosphofructokinase activity to its physiological range. If the results of our present in vitro studies apply in vivo, it is possible that the 10% phosphofructokinase bound to the membrane, which has lost its sensitivity to ATP and 2,3-diphosphoglycerate is sufficiently active to account for the observed phosphofructokinase activity in the red cell.

Although phosphofructokinase, as well as aldolase and glyceraldehyde-3-P dehydrogenase, is dissociated from ghosts by washing with 150 mM NaCl, which is close to the osmolarity of erythrocyte, it is possible that different conditions inside the cell such as higher phosphofructokinase concentration, binding of Cl⁻ to other proteins and the presence of effectors favor the binding of phosphofructokinase. The effect of salt and various effectors on the binding is currently under investigation.

It is interesting to note that a similar loss of sensitivity to ATP inhibition was also observed with phosphofructokinase which has been covalently linked to cyanogen bromide-activated Sepharose. This covalent linkage was through lysine residues of phosphofructokinase. Clearly, the modes of binding of phosphofructokinase to Sepharose and that to the erythrocyte membranes are different. However, both may involve an interaction, whether ionic or covalent, of the same residues at the allosteric site of phosphofructokinase with different surfaces and may result in a fixed (or frozen) conformation.

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