Organization of enzymes in human erythrocyte membranes

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Studies were directed at determining whether there was a specific organization of enzymes in erythrocyte membranes. Membranes prepared in the absence of Mg++ which were disrupted by exposure to sonic vibrations or lipid-active agents underwent an increase in activity of phosphoglycerate kinase but not glyceraldehyde phosphate dehydrogenase. When membranes which were prepared in the presence of Mg++ were disrupted by sonic oscillations and lipid-active agents, there were relatively large increases in activity of both phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase. The data are interpreted as showing that membrane phosphoglycerate kinase was oriented toward the lipid core of the membrane, whereas membrane glyceraldehyde phosphate dehydrogenase was directed toward the interior of the erythrocyte. It is postulated that this organization of enzymes in erythrocyte membranes might function by providing ATP for pump ATPase.

The experiments to be described were devised to determine if there is an organization of enzymes in human erythrocyte membranes which could have importance in membrane transport and in the pathogenesis of hemolytic disease (15). We had previously reported the preparation of morphologically intact human erythrocyte membranes which retained 0.03 % of the original hemoglobin content, and which contained a highly specific complement of tightly bound glycolytic enzymes, of which glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase (forward assay) were present in highest concentration (16). Levels of membrane glyceraldehyde phosphate dehydrogenase (GAPD) were 580 μmoles/hr per 1011 membranes in contrast to whole hemolysate values of 3,500 μmoles/hr per 1011 RBC, and comparable values for phosphoglycerate kinase (PGK) (forward assay) were 80 μmoles/hr per 1011 membranes and 80,000 μmoles/hr per 1011 RBC. These membranes contained all of the erythrocytic lipid (unpublished observations), and membranes with similar properties have been prepared in several laboratories (2, 19). The experiments were directed at determining where in the membrane the enzymes GAPD and PGK were located, because a result of their coupled catalytic activity could be the production of ATP at or near the membrane.

The approach to the dissection of morphologically intact membranes was based on a membrane model in which a bimolecular layer of lipid is enclosed between two layers of protein (13, 17) and the negative charge is supplied by neuraminic acids (3). Mg++ functions in some way to preserve membrane cohesion (7). Membranes prepared both in the absence and in the presence of Mg++ were therefore exposed to ultrasonic vibration, trypsin, bromelin, digitonin, lecinthinase (phospholipase C), and neuraminidase. Following exposure of membranes to these agents, measurements were made of the membrane enzymes, GAPD and PGK.

MATERIALS

The following materials were obtained from manufacturers: DPN, DPNH, ATP, ADP, 2,3-diphosphoglycerate, 3-phosphoglycerate, fructose diphosphate, DL-glyceraldehyde-3-phosphate, phospholipase C (lecithinase), and neuraminidase V from Sigma Chemical Co., St. Louis, Missouri; ribose-5-phosphate from Nutritional Biochemicals Corp., Cleveland, Ohio; 2-mercaptoethanol, thiamine pyrophosphate, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycric kinase, triose phosphate isomerase, α-glycerophosphate dehydrogenase from Calbiochem, Los Angeles, California; bovine serum albumin from Armour Pharmaceutical Corp., Kankakee, Illinois; bromelin from Dade Reagents, Inc., Miami, Florida; and trypsin from Difco Lab, Detroit, Michigan.

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1This work was supported by Grant HE-05305 from the National Heart Institute. Preliminary results were reported to the IXth Congress of the International Society of Hematology (15).
2Markle Scholar in Academic Medicine.
TABLE 1. Enzyme assays on standard and Mg++ membranes

<table>
<thead>
<tr>
<th>Enzymes Assayed</th>
<th>Treatment to Which Membranes Were Subjected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sonic disruption</td>
</tr>
<tr>
<td>GAPD</td>
<td>68±1.0*</td>
</tr>
<tr>
<td>PGK, forward</td>
<td>138±3.2</td>
</tr>
<tr>
<td>PGK, backward</td>
<td>123±6.0</td>
</tr>
</tbody>
</table>

A: standard membranes

B: Mg++ membranes

GAPD 238±10* 317±28* 241±27 244±19 245±18 99±5.0 97±9.0
PGK, forward 230±12 241±27 244±19 82±3.6 80±4.0 84±3.0
PGK, backward 200±27 228±30 265±35 101±6.0 86±3.6 92±10

Results are expressed as the mean ± 3σ of the ratio of the enzyme activity of treated membranes to the activity of control preparations, which was arbitrarily set at 100. Standard and Mg++ membranes were individually controlled and each form of treatment had its own control (see methods). We are indebted to Dr. Lincoln Moses, Professor of Biostatistics, Stanford University, and his staff for statistical analysis of the data. Numbers in parentheses indicate the number of experiments performed. * Results of GAPD assays after sonic disruption of membranes were corrected for the mean 25% inhibition induced by exposure of soluble purified GAPD to sonic vibration (see methods).

TABLE 2. Membrane enzyme activities

<table>
<thead>
<tr>
<th>Membranes Disrupted by Sonic Oscillation</th>
<th>Aldolase</th>
<th>Transketolase</th>
<th>Phosphoribosylpyrophosphate-synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>91 (2)</td>
<td>101 (3)</td>
<td>103 (3)</td>
</tr>
</tbody>
</table>

Mean enzyme activity of membranes after sonic disruption was related to the activity of whole membranes which was arbitrarily set at 100. Numbers in parentheses are the number of experiments performed.

METHODS

A. Preparation of Red Cell Membranes

Erythrocyte membranes were prepared as previously described (16), with a single modification. In the second dialysis, tris (hydroxyxymethyl) aminomethane-buffered bovine serum albumin (pH 7.0) was added to the membrane suspension to give a final albumin concentration of 0.5 mg/ml. In the third, fourth, and fifth dialyses, sufficient bovine serum albumin was added to give a final concentration of 0.25 mg/ml of membrane suspension. Membranes were quantified in the Coulter counter model A, and the electronically obtained counts were regularly confirmed by hand-counting, using phase microscopy.

For the preparation of erythrocyte membranes in the presence of Mg++, the identical procedure was followed with the addition of 0.25 mM MgCl₂ to the dialyzing solution. Membranes prepared in the absence of Mg++ will be called standard membranes, whereas those prepared in the presence of Mg++ will be called Mg++ membranes. Membrane suspensions contained 4.5 X 10⁸ membranes/ml in an isotonic chloride solution composed of 146 mM sodium, 5.1 mM potassium, and 2 mM Mg++, and were buffered to pH 7.5 with 0.00017 M tris.

Determinations of membrane hemoglobin content were made by the methods of Proc. Mitchell, and Hanahan (2).

The hemoglobin content was 0.028 mg/ml of standard membranes and 0.032 mg/ml of Mg++ membranes.

B. Treatment of Erythrocyte Membranes

All the agents which were used to "dissect" erythrocyte membranes were tested against suitably diluted preparations of soluble purified GAPD and PGK to check for inactivation caused by that form of treatment. With a single exception, the highest concentration of reagent which did not cause inactivation of the soluble purified enzymes was used. Exposure of soluble purified muscle GAPD to sonic oscillation for 8 min resulted in complete inactivation. The addition of 2.0 μmoles/ml of 2-mercaptoethanol during exposure to sonic oscillations preserved an average of 70% of the GAPD activity. Sonic oscillations produced no changes in soluble yeast PGK. Therefore these conditions of exposure to sonic vibrations were used even though they resulted in an average of 25% inhibition of the soluble GAPD. The appearance of membranes under the phase microscope after exposure to the various agents was recorded. In each case a control membrane preparation was treated in an identical manner in terms of time, temperature, pH, and electrolyte composition, leaving only the factor to be tested as the variable.

1. Sonic oscillation. Exposure of erythrocyte membranes to sonic oscillation was performed in a 10-ke Raytheon sonic oscillator in polypropylene tubes of 8 mm inner diameter containing 1.0 ml of membrane suspension and 2 μmoles of 2-mercaptoethanol during exposure to sonic oscillations preserved an average of 75% of the GAPD activity. Sonic oscillations produced no changes in soluble yeast PGK. Therefore these conditions of exposure to sonic vibrations were used even though they resulted in an average of 25% inhibition of the soluble GAPD. The appearance of membranes under the phase microscope after exposure to the various agents was recorded. In each case a control membrane preparation was treated in an identical manner in terms of time, temperature, pH, and electrolyte composition, leaving only the factor to be tested as the variable.

2. Digitonin. One milliliter of erythrocyte membrane suspension containing 4.5 X 10⁸ membranes was incu-
C. Enzyme Assays

Assays for membrane enzymes were performed on portions of the treated and control suspensions. Reactions were carried out in 1-cm light path Beckman cuvettes of 3-ml capacity. Turbidity was minimal and could be controlled by using appropriate blanks. Since assays involved interconversions of oxidized and reduced pyridine nucleotide coenzymes, the optical density change at 340 μμ was recorded and millimolar extinction coefficient of 6.22 was used for calculations.

1. a) Glyceraldehyde phosphate dehydrogenase was assayed as previously described (16) with the following changes in concentration of reactants. Three-milliliter reactions mixtures contained: tris buffer, pH 7.5, 165 μμoles; d-glyceraldehyde-3-phosphate, 3.3 μμoles; sodium arsenate, 30 μμoles; 2-mercaptoethanol, 20 μμoles; DPN, 4.0 μμoles; 0.01 ml membrane suspension (2.45 X 10^9 membranes/ml). b) Reverse GAPD assay was performed by the method of Wu and Racker (21): 3.0-ml reaction mixtures contained: tris buffer, pH 7.5, 100 μμoles; MgCl₂, 15 μμoles; 2-mercaptoethanol, 60 μμoles; 3-phosphoglycerate, 10 μμoles; DPNH, 0.6 μμoles; ATP, 4 μμoles; 10 μg PGK (capable of converting 1.8 μμoles substrate/min); 0.02-ml membrane suspension (2.45 X 10^9 membranes/ml). Contamination of PGK with GAPD was less than 0.005%.

2. Phosphoglycerate kinase was measured by the “forward” and “backward” assays as previously described with modifications of reactants as follows (16). a) The “forward” assay contained in a volume of 5.0 ml: tris buffer, pH 7.5, 165 μμoles; MgCl₂, 15 μμoles; 2-mercaptoethanol, 20 μμoles; inorganic phosphate buffer, pH 7.5, 60 μμoles; DPN, 4.0 μμoles; ADP, 5.0 μμoles; d-glyceraldehyde-3-phosphate, 3.3 μμoles; glyceraldehyde phosphate dehydrogenase, 0.017 mg/ml (capable of converting 0.6 μμoles/ml of substrate/min); 0.1 ml membrane suspension (2.45 X 10^9 membranes/ml). b) The “backward” assay contained in a volume of 9.0 ml: tris buffer, pH 7.5, 100 μμoles; MgCl₂, 15 μμoles; 2-mercaptoethanol, 60 μμoles; DPNH, 0.6 μμoles; 3-phosphoglycerate, 10 μμoles; ATP, 4.0 μμoles; glyceraldehyde phosphate dehydrogenase, 0.017 mg/ml; 0.2-ml membrane suspension (2.45 X 10^9 membranes/ml).

3. Diphosphoglyceromutase was assayed by the method of Raoport and Luebering (12). Concentration of reactants was identical to that described for the forward phosphoglycerate kinase assay except that ADP was omitted.

4. Assays of membrane transketolase, aldolase, and phosphoribosomerase were performed as previously described (16).

RESULTS

A. Enzyme Assays in Erythrocyte Membranes

1. Membranes prepared in the absence of magnesium (standard membranes). The results of backward and forward PGK assays and GAPD assays in control and treated erythrocyte membranes prepared in the absence of Mg⁺⁺ are summarized in Table 1. A. None of the measures altered GAPD activity. The 32% decrease in membrane GAPD activity induced by sonic oscillations was not different.

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We are grateful to Dr. F. W. Michel, Dept. of Medicine, Stanford Medical School, for performing these assays for us.
TABLE 4. Enzyme assays on mixtures of whole and disrupted membranes and soluble enzymes

<table>
<thead>
<tr>
<th>Vol of Diluted Membrane Suspension or Enzyme Solution Added to 3.0 ml Reaction</th>
<th>Whole Membranes</th>
<th>Disrupted Membranes</th>
<th>Diluted Soluble Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPD assay, 5-min incubation at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µl</td>
<td>0.020*</td>
<td>0.050</td>
<td>0.025</td>
</tr>
<tr>
<td>100 µl</td>
<td>0.025</td>
<td>0.065</td>
<td>0.069</td>
</tr>
<tr>
<td>150 µl</td>
<td>0.066</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>PGK assay (forward), 15-min incubation at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µl</td>
<td>0.025</td>
<td>0.050</td>
<td>0.028</td>
</tr>
<tr>
<td>100 µl</td>
<td>0.060</td>
<td>0.110</td>
<td>0.120</td>
</tr>
<tr>
<td>150 µl</td>
<td>0.090</td>
<td>0.165</td>
<td></td>
</tr>
<tr>
<td>PGK assay (backward), 30-min incubation at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µl</td>
<td>0.040</td>
<td>0.055</td>
<td>0.040</td>
</tr>
<tr>
<td>200 µl</td>
<td>0.080</td>
<td>0.115</td>
<td>0.085</td>
</tr>
<tr>
<td>300 µl</td>
<td>0.125</td>
<td>0.155</td>
<td></td>
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Mixtures†

<table>
<thead>
<tr>
<th>GAPD</th>
<th>PGK Forward</th>
<th>PGK Backward</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>Calculated</td>
<td>Observed</td>
</tr>
<tr>
<td>1 Part whole membranes plus 1 part disrupted membranes</td>
<td>0.045</td>
<td>0.050</td>
</tr>
<tr>
<td>2 Vol whole membranes plus 1 vol disrupted membranes</td>
<td>0.065</td>
<td>0.065</td>
</tr>
<tr>
<td>1 Part whole membranes plus 2 parts disrupted membranes</td>
<td>0.075</td>
<td>0.085</td>
</tr>
<tr>
<td>2 Parts whole membranes plus 1 part soluble enzyme</td>
<td>0.075</td>
<td>0.070</td>
</tr>
<tr>
<td>2 Parts disrupted membranes plus 1 part soluble enzyme</td>
<td>0.100</td>
<td>0.100</td>
</tr>
</tbody>
</table>

The data in this table are from a single experiment. Two similar experiments gave essentially the same result. Membranes exposed to sonic vibration are called disrupted membranes. * Numbers indicate the AOD at 340 mp. † Mixtures were preincubated in indicated proportions for 15 min at room temperature. ‡ In the GAPD and forward PGK assays 1 part is 50 µl, whereas in the backward PGK assay 1 part is 100 µl.

from the 25% decrease seen when soluble muscle GAPD solutions were treated with sonic vibrations (see methods). In contrast, there was an increase in PGK seen in both assays after membranes were exposed to sonic oscillations. Assays for transketolase, aldolase, and phosphoribosilomerase were performed on membranes before and after exposure to sonic oscillations (Table 2) and there was no change in activity. Therefore an increase in PGK activity, measurable in both assays, was seen following sonic disruption of membranes and exposure of membranes to digitonin and lecithinase.

2. Membranes prepared in 0.25 mM Mg++ (Mg++ membranes). The results are summarized in Tables 1, B, and 3.

a. Whole untreated Mg++ membranes had less GAPD activity but more PGK activity than did whole standard membranes (Table 3). The maximum GAPD and PGK contents of Mg++ membranes, determined after either sonic disruption, digitonin, or lecithinase, were greater than standard membranes (Tables 1, B, and 3).

b) There was a threefold increase in GAPD activity of Mg++ membranes after exposure to sonic vibration, digitonin, and lecithinase (Table 1, B), whereas these measures produced no changes in standard membranes (Table 1, A).

c) Sonic disruption, digitonin, and lecithinase produced much larger increases in PGK activity of Mg++ membranes as contrasted with standard membranes (Table 1, B).

d) Trypsin, bromelin, and neuraminidase may have had inhibitory effects on PGK activity of Mg++ membranes in the forward assay (Table 1, B).

B. Increase in Membrane PGK Activity

Other than the uncovering of active sites of enzymes, there are several possible explanations for the observed increase in membrane PGK activity which followed on sonic disruption of standard and Mg++ membranes, and for the increase in GAPD seen after sonic disruption of Mg++ membranes.

1. Sonic vibrations of membranes could have induced a release of interfering enzymes which might have
ENZYMES IN ERYTHROCYTE MEMBRANES

TABLE 5. GAPD assays (expressed as μmoles/hr per 10^11 membranes)

<table>
<thead>
<tr>
<th></th>
<th>Standard Membranes</th>
<th>Mg++ Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole</td>
<td>After sonic</td>
</tr>
<tr>
<td>Forward assay</td>
<td>945</td>
<td>935</td>
</tr>
<tr>
<td>Reverse assay</td>
<td>935</td>
<td>905</td>
</tr>
</tbody>
</table>

Data are from a single experiment. Results of standard membranes after sonic disruption were corrected for the mean 25% inhibition induced by exposure of soluble purified GAPD to sonic vibration. Two similar experiments gave essentially the same results.

falsely distorted the PGK assays. Of the enzymes known to be present in RBC only diphosphoglyceromutase (DGM) could have caused higher values for PGK in the forward assay, but it should then have given falsely lower values in the backward PGK assay. However, sonic disruption of membranes produced PGK increases of similar magnitude in both forward and backward assays (Table 1). Direct assays on whole and sonicated standard membranes for diphosphoglyceromutase revealed no measurable activity (less than 0.5 μmoles/hr per 10^11 membranes).

2. Sonic vibrations of membranes could have released activators or destroyed inhibitors of PGK or GAPD. To investigate this possibility, whole Mg++ membranes and Mg++ membranes exposed to sonic oscillations were incubated with each other and with their respective soluble purified enzyme in varying combinations and the results were compared with the anticipated arithmetic sum. There was no potentiation of activity by sonicated membranes and no depression of activity by whole membranes for either enzyme tested (Table 4).

3. The increase in PGK activity (but not GAPD activity) in standard and Mg++ membranes could be explained by a special impermeability of membranes to 1,3-diphosphoglycerate. Sonic disruption of membranes could then have resulted in apparently higher PGK values simply by allowing 1,3-diphosphoglycerate to reach the indicator enzyme, GAPD, in the supernatant solution. This possibility was explored by measuring GAPD activity of whole and disrupted membranes in the reverse assay (see METHODS), in which the substrate 1,3-diphosphoglycerate is generated in the supernatant medium. If membrane impermeability prevented 1,3 diphosphoglycerate from reaching the active sites on the GAPD molecule, the reverse GAPD assay on whole standard and Mg++ membranes would give lower results than the forward assay in which glyceraldehyde-3-phosphate is the substrate. Reverse and forward GAPD assays (Table 5) on whole and disrupted standard and Mg++ membranes yielded entirely comparable results.

FIG. 1. Hypothetical erythrocyte membrane showing four possible orientations of an enzyme molecule within the protein coat.

Both standard and Mg++ membranes were stored after preparation, for 60 min, in a solution containing 2.0 mM Mg++. However, only the membranes actually hemo-
lyzed and prepared in 0.25 mM Mg++ showed the Mg++ effect. The GAPD activity of whole untreated Mg++ membranes was less than that of whole untreated stand-
ard membranes (Table 3), probably because the substrates could not penetrate to the enzyme sites which are within the membrane. Sonic disruption of Mg++ membranes and exposure to lipid-active agents did result in striking increases in GAPD activity of Mg++ membranes, probably by allowing substrates to penetrate to the enzyme. The results with Mg++ membranes reinforce the idea of an interior location of GAPD but do not distinguish between orientations 2, 3, or 4. Combining the data with standard and Mg++ membranes, GAPD would be at orientation 4. There should then be no GAPD measurable in whole Mg++ membranes; however, it is probable that even in the Mg++ preparation there are populations of more permeable membranes which account for the small activity observed, and this finding has been previously noted (6).

Standard membranes are freely permeable to the substrates used in the enzyme assays (18); however, there was a significant increase in PGK activity after exposure of membranes to digitonin, lecithinase, and sonic vibration. Therefore, it is more likely that PGK is at orientations 2 or 3 since the increase in activity cannot be explained by increased permeability of substrates. The finding that any PGK can be assayed in whole standard membranes again suggests the existence of several populations of membranes, some of which were so altered by the preparation that even the lipid core was opened. The observation of large increases in PGK activity of Mg++ membranes after exposure to sonic vibrations, lecithinase, and digitonin emphasizes the inner location of membrane PGK.

It is not clear why untreated Mg++ membranes had greater PGK activity than standard membranes. Mg++ membranes had approximately 14% more hemoglobin than standard membranes (see Methods), and it is possible that Mg++ induced the adsorption of hemolysate enzymes to membranes.

The data can be interpreted as showing that there is an organization of enzymes in the membrane such that GAPD can obtain substrates from the cell interior (the inorganic phosphate may come either from the inside or outside (1)) and convert them to 1,3-diphosphoglycerate. The 1,3-diphosphoglycerate is then available to PGK which is oriented toward the core and conversion to ATP can be completed (Fig. 2). The proposed scheme for localization of GAPD and PGK in the membrane indicates a possible source of substrate, ATP, for membrane ATPase. The association between membrane ATPase and active Na+-K+ transport is well known (11).

There is evidence that ATPase is located within the membrane. Its demonstration usually requires some disruptive attack on the membrane (9). ATPase was not inactivated by exposure to trypsin and bromelin in concentrations which inactivated membrane acetylcholinesterase (5). However, exposure to lecithinase inactivated erythrocyte membrane ATPase, a finding which suggests that an intact lipid core is somehow necessary for optimum catalytic activity (14).

There are other possible interpretations of the data; however, irrespective of the membrane model used (20), only PGK of the five membrane enzymes tested appears to be oriented toward the interior of the membrane. The difference between PGK and GAPD and the other membrane enzymes is consistently demonstrated in two assays, and differences in membrane impermeability of substrates cannot account for the changes observed. This finding alone indicates that there may be an organization of enzymes in human erythrocyte membranes.

We are grateful to Dr. Paul Berg, Department of Biocenetics, and Dr. William P. Creger, Department of Medicine, Stanford Medical School, for their critical review of the manuscript and for their free discussions of the experimental work.

REFERENCES


