Association of Glyceraldehyde 3-Phosphate Dehydrogenase with the Human Erythrocyte Membrane

EFFECT OF DETERGENTS, TRYPsin, AND ADENOSINE TRIPHOSPHATE*

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SUMMARY

Glyceraldehyde 3-phosphate dehydrogenase is one of the major protein components associated with the erythrocyte membrane. This has been shown by activity studies of specific membrane extracts and by the specificity of iodoacetate inactivation and labeling of the enzyme. Studies on the association of the enzyme with the membrane are complicated by the tendency of the ghosts to seal under certain conditions, thus rendering the enzyme inaccessible to its substrates. This crypticity of the enzyme activity can be eliminated by detergent treatment. Sodium dodecyl sulfate is preferred, since at low concentrations it causes only minor decreases in the enzyme activity, while Triton X-100 gives more extensive inactivation at all concentrations tested. Trypsin digestion can release glyceraldehyde d-phosphate dehydrogenase from the membrane, but it also causes a fraction of the ghosts to seal. The fraction which reseals is somewhat variable between different membrane preparations and is related to the prior treatments of the membranes.

Under standard hypotonic hemolysis conditions (10 mM Tris), 60 to 80% of the glyceraldehyde 3-phosphate dehydrogenase remains associated with the membrane. Aldolase exhibits similar behavior, while lactic dehydrogenase and hemoglobin are almost completely released. In the presence of ATP (1 mM) the amount of glyceraldehyde 3-phosphate dehydrogenase associated with the membrane is reduced to 5 to 20%. EDTA, Ca++, Mg++, and 2,3-diphosphoglycerate show little effect at concentrations of 1 mM, although release of the enzyme is promoted by these agents at higher concentrations tested. At 0°, solubilized glyceraldehyde 3-phosphate dehydrogenase is inactivated by ATP, a process that can be prevented if the enzyme is preincubated with NAD or if the incubation with ATP is performed at 25°. The ATP-promoted release of glyceraldehyde 3-phosphate dehydrogenase during hemolysis is not altered by preincubation with NAD or by raising the temperature to 25°. Therefore, the two phenomena (enzyme inactivation and release from the membrane) appear to be unrelated. It is postulated that the effect of ATP on enzyme release is due to a direct action of ATP on the membrane.

The role and importance of enzyme-membrane associations has received considerable attention in recent years. Membrane-bound enzymes can be divided into two categories, integral and peripheral, which correspond approximately to the definitions of Singer and Nicolson (1). Integral enzymes, such as the ATPases and acetylcholinesterase of the erythrocyte membrane, are very difficult to dissociate from the membrane without inactivation of the enzyme and gross membrane distortion or destruction. These enzymes often require lipid for activity. The peripheral enzymes are more loosely bound and can be dissociated by relatively mild treatments, e.g. extraction with salt, which do not destroy membrane structure. The peripheral enzymes also exhibit a wide range of behaviors in their associations with membranes, as shown by the study of Duchon and Collier (3) on the association of a series of enzymes with erythrocyte membranes. It seems clear that enzymes can associate with membranes in a variety of modes, and that a variety of such associations will have to be studied to understand the factors which might be involved for different enzymes.

In an earlier study we found that one of the major polypeptide components of the human erythrocyte membrane was labeled specifically by iodoacetate (4). This component was later identified as a subunit of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.23; n-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating)) on the basis of the enzyme activity of the purified component, specific inhibition of the enzyme by iodoacetate, and a comparison with the results of Tanner.

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and Gray (5), who identified the same membrane component by a partial amino acid sequence analysis. Two factors relating to the association of glyceraldehyde 3-phosphate dehydrogenase with the erythrocyte membrane were of particular interest. First, the enzyme of isolated membranes was not attacked readily by proteolytic enzymes (6, 7), but was released into the supernatant during the course of digestion (6). Second, the addition of ATP to the red cells during hemolysis resulted in a dramatic decrease in the enzyme isolated with the membrane as measured by iodoacetate labeling. The latter discovery was of particular interest because of the known effects of ATP on the activity and quaternary structure of glyceraldehyde dehydrogenases from rabbit muscle and yeast (8-10). The present paper describes the investigation of the effects of ATP and trypsin on the association of the enzyme with the human erythrocyte membrane.

EXPERIMENTAL PROCEDURES

Materials and Methods—Human blood was obtained from the Dallas Community Blood Bank and used within 1 week of the withdrawal date. Washed erythrocytes, resealed ghosts, and isolated membranes were prepared as previously described (4). All enzymes and chemicals for enzyme assays were products of Sigma Chemical Company. Chemicals for electrophoresis were obtained from Eastman (highest purity grade) or Ciba. Other chemicals were reagent grade or highest purity available.

Hemoglobin (11), protein (12), phosphorus (13), and cholesterol (14) were determined by established procedures. The enzymes glyceraldehyde 3-phosphate dehydrogenase (15, 16), lactic dehydrogenase (17), acetylcholinesterase (18), and aldolase (15, 16) were assayed by previously described procedures. Iodoacetate labeling and SDS-acrylamide electrophoresis were performed as previously described (4). Bands on electrophoresis are labeled as previously described (4).

Molecular Weight Determination of Extracted Glyceraldehyde 3-Phosphate Dehydrogenase—The enzyme was extracted from isolated membranes with NaCl (4) or EDTA (3). The crude extracts were dialyzed against 50 mM glycine, 10 mM Tris, and 5 mM mercaptoethanol (pH 9.2) at 4° overnight. After concentration by Aquacide III the enzyme was dialyzed into 250 mM glycine, 5 mM mercaptoethanol, and 1 mM EDTA at 4° and loaded onto a Sephadex G-200 column (2.5 x 90 cm) equilibrated with the same buffer. Fractions of 3.8 ml were collected and assayed for protein, hemoglobin, acetylcholinesterase, and glyceraldehyde 3-phosphate dehydrogenase. Fractions containing the latter two activities were combined and assayed for phosphorus and cholesterol. The molecular weight of the dehydrogenase was estimated from a calibration curve for the column using catalase, aldolase, lactic dehydrogenase, ovalbumin, chymotrypsinogen, lysozyme, and cytochrome c as molecular weight standards. For sucrose density gradient centrifugation the crude EDTA extract was precipitated in a 60 to 90% ammonium sulfate fraction and dialyzed into 50 mM Tris and 2 mM mercaptoethanol (pH 7.4). Samples of 200 µl plus 50 µl of molecular weight standard were mixed, and 100 µl of the mixture were applied to 5 to 20% buffered sucrose gradients. These were centrifuged 17 hours at 4° and 114,000 x g in a Beckman L-2 ultracentrifuge with an SW 50.1 head. The molecular weight was estimated by the procedure of Martin and Ames (19), using catalase, lysozyme, and lactic dehydrogenase as standard proteins.

Detergent Effects on Membrane Glyceraldehyde 3-Phosphate Dehydrogenase—One volume of detergent solution (Triton X-100 or SDS) in an appropriate medium was mixed with 1 volume of packed ghosts. At timed intervals, 50 µl of the mixture were taken and immediately assayed for glyceraldehyde 3-phosphate dehydrogenase activity. Similar procedures were used to determine the detergent effects on aldolase and acetylcholinesterase activities. The molecular weight of Triton X-100 was taken as 680.

Trypsin Digestion of Erythrocyte Membranes—Isolated erythrocyte membranes were digested with trypsin at an appropriate concentration at room temperature in 7 mM phosphate (pH 7.4). At timed intervals three samples were removed. (a) An aliquot was assayed directly for glyceraldehyde 3-phosphate dehydrogenase activity; (b) an aliquot was solubilized by mixing with one-fourth volume of 10% SDS-5% mercaptoethanol and used for SDS electrophoresis; and (c) an aliquot was treated with soybean trypsin inhibitor and centrifuged. The supernatant and pellet fractions of this sample were assayed for enzyme activities. In some experiments pellets were subjected to a freeze-thaw or SDS treatment before assay. The mixture from the freeze-thaw disruption was centrifuged, and the pellet and supernatant were assayed again for glyceraldehyde 3-phosphate dehydrogenase.

Addition of Membrane Effectors during Hemolysis—One volume of washed, packed erythrocytes was hemolyzed in 10 to 12 volumes of 10 mM Tris (pH 7.4) containing appropriate concentrations of ATP, EDTA, 2,3-diphosphoglycerate, or divalent cations at 0° for 20 min. The mixture was centrifuged at 25,000 x g for 20 min at 4° and samples of the supernatant and pellet were dialyzed against 10 mM Tris (pH 7.4) and 2 mM mercaptoethanol at 4° overnight. Supernatant and pellet samples were assayed for hemoglobin and enzymes. The dialyzed pellets were pre-treated with SDS before assay. Activities released into the supernatant are expressed as a percentage of the total activity (supernatant plus pellet). These total activities did not deviate significantly from untreated controls as a result of treatment with the various effectors.

Effect of ATP or NAD on Solubilized Glyceraldehyde 3-Phosphate Dehydrogenase—Ito samples of the crude EDTA extract of the erythrocyte membrane at 0° or 25° was added ATP to 1 mM or NAD to 2 mM. After 10 min this was followed by addition to the appropriate samples of NAD to 2 mM or ATP to 1 mM. After an additional 30 min of incubation aliquots were assayed for glyceraldehyde 3-phosphate dehydrogenase.

To determine effects of chymotrypsin on erythrocyte glyceraldehyde 3-phosphate dehydrogenase, a 60 to 80% ammonium sulfate fraction of the crude EDTA extract was used. The crude dehydrogenase preparation (0.17 mg per ml) was incubated with chymotrypsin (10 µg per ml) in 60 mM Tris (pH 7.4) and 1.5 mM mercaptoethanol in the presence or absence of 5 mM ATP, 5 mM NAD, or both at room temperature. Aliquots were removed at timed intervals for dehydrogenase assay.

RESULTS

Characterization of Glyceraldehyde 3-Phosphate Dehydrogenase—In previous studies only a single protein of the human erythrocyte membrane was shown to be labeled by iodoacetate (4). This protein could be extracted from the membrane with 0.5 M sodium chloride. Subsequent activity measurements and iodoacetate inactivation studies indicated that the protein was the enzyme glyceraldehyde 3-phosphate dehydrogenase. This same protein was extracted with 0.1 M EDTA from human erythrocyte membranes by Tanner and Gray (5), who established its identity by...
partial amino acid sequence analysis and activity studies. As a further characterization of the erythrocyte enzyme, some other parameters of the membrane extracts have been investigated using both sodium chloride and EDTA as extractants. Table I shows analytical data on extracts obtained from isolated membranes by the two different procedures. Both methods have shown considerable variation in the amounts of protein and lipid extracted from different membrane preparations. This is due in part to the variation in the membrane preparations themselves, which contain variable amounts of hemoglobin and other extractable proteins. The bulk of the protein extracted by either method is the hemoglobin and glyceraldehyde dehydrogenase, as shown earlier by electrophoresis (4). Smaller amounts of other proteins are also extracted. The EDTA extraction routinely yields more consistent results in that smaller amounts of lipid and other proteins are extracted with the glyceraldehyde 3-phosphate dehydrogenase.

As a result the specific activity of the enzyme is higher. The large amount of lipid associated with the NaCl extract of Table I suggested that the enzyme glyceraldehyde 3-phosphate dehydrogenase may also be able to interact with lipid to some extent in binding to the membrane. Therefore, a sample of the extract was chromatographed on Sephadex G-200 to determine the size of the enzyme isolated and the presence of lipid. Fig. 1 shows that the glyceraldehyde 3-phosphate dehydrogenase activity elutes as a broad peak. The molecular weight calculated from a column calibration curve is 150,000, consistent with the monomeric weight of 35,000 to 37,000 found by SDS electrophoresis (4) and a tetrameric native structure. The broadness of the peak and the skew toward lower molecular weight suggest that some dissociation may occur in this system. The native molecular weight (150,000) was confirmed by sucrose density gradient centrifugation, which gave a sharper, more symmetrical peak. Acetylcholinesterase activity is eluted at the void volume of the column. All of the detectable lipid eluted from the column was present in this fraction. SDS electrophoresis indicated that the major protein eluted in this excluded fraction was Component I (spectrin), the large polypeptide component of the red cell membrane (20). This does not imply that spectrin associates with lipid or acetylcholinesterase, but merely that both are aggregated under the conditions of chromatography. The association of acetylcholinesterase and lipid does seem likely, since it has been previously shown by Burger et al. (21) for bovine erythrocyte membrane acetylcholinesterase. We have also noted that increased salt extraction of acetylcholinesterase is paralleled by increased lipid release from the membrane. This behavior would be expected of a membrane enzyme which is associated with the membrane lipid. Both column chromatography and sucrose density gradient centrifugation indicate that the glyceraldehyde 3-phosphate dehydrogenase is extracted from the membrane in a nonaggregated form with a native molecular weight similar to that observed for this enzyme from other sources, while the acetylcholinesterase is released in an aggregated form with lipid.

The reaction of iodoacetate with erythrocyte membranes of intact erythrocytes is strongly directed toward a single protein. That this specificity is due to a highly specific reaction of glyceraldehyde 3-phosphate dehydrogenase can be seen from Fig. 2. The incorporation of the label into the membrane closely parallels glyceraldehyde 3-phosphate dehydrogenase activation. Two other interesting facets of the iodoacetate reaction with erythrocytes were noted. The reaction with the intracellular soluble proteins (primarily hemoglobin) is multiphasic and the activity of membrane acetylcholinesterase is enhanced by the iodoacetate treatment. A similar enhancement of membrane-bound aldolase activity was also noted under similar conditions. The high reactivity of glyceraldehyde 3-phosphate dehydrogenase relative to the other membrane proteins and hemoglobin suggested that it might occupy a unique location along or near an anion permeation pathway in the membrane. Therefore labeling experiments were conducted on cells which had been broken by freezing and thawing to eliminate the membrane barrier. No differences were noted in iodoacetate incorporation into either hemoglobin or glyceraldehyde 3-phosphate dehydrogenase between the intact and broken cells. Therefore, it appears that the specificity of the reaction is due to the high affinity of the iodoacetate for the glyceraldehyde 3-phosphate dehydrogenase, with the reagent acting as an affinity label because of the presence of positively charged NAD at the active site, as indicated by Fersht (22).

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<tr>
<td>phosphate dehydrogenase</td>
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<td>Lactic dehydrogenase</td>
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<tr>
<td>Cholinesterase</td>
<td>20</td>
<td>5.7</td>
</tr>
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</table>

Fig. 1. Column chromatography of NaCl extract of erythrocyte membranes on Sephadex G-200. Isolation and chromatography are described under "Experimental Procedures." Combined fractions from the column were analyzed for phosphorus and cholesterol and subjected to SDS acrylamide electrophoresis. Acetylcholinesterase (O—O), glyceraldehyde 3-phosphate dehydrogenase (●—●), and hemoglobin (△—△).
Incorporation of iodoacetate into membrane and hemoglobin of intact erythrocyte and its effect on glyceraldehyde 3-phosphate dehydrogenase and acetylcholinesterase. Reaction was performed with 100 μM [14C]iodoacetate at 25°C as previously described (4). Acetylcholinesterase activity (O---O), glyceraldehyde 3-phosphate dehydrogenase activity (●—●), hemoglobin labeling (△—△), and membrane labeling (●—△). Initial activity of acetylcholinesterase was 0.70 μmole per min per mg of membrane protein and for glyceraldehyde 3-phosphate dehydrogenase was 0.72 μmole per min per mg of protein.

During the course of experiments on the activity of glyceraldehyde 3-phosphate dehydrogenase in erythrocyte membranes, it was found that the enzyme activity decreased dramatically under certain conditions of incubation. This activity loss was shown to be due to rescaling of the ghosts such that they were impermeable to the enzyme substrate. Proteolysis of the ghosts is a good indicator of whether rescaling has occurred, since the proteins accessible to proteolysis are different in the sealed and unsealed ghosts (7). With this prior knowledge it is also now possible to use glyceraldehyde 3-phosphate dehydrogenase activity as a monitor for the sealing of the membranes, although it is probably not an absolute marker because of some leakage of substrates into the membranes. In order to investigate the association of the dehydrogenase with the membrane, it is necessary to eliminate the effect of membrane rescaling on the activity. Since this is most readily accomplished by detergent treatment, the effects of two different common detergents on the dehydrogenase activity were investigated.

The effects of sodium dodecyl sulfate and Triton X-100 on the dehydrogenase enzyme activity of freshly prepared erythrocyte ghosts are shown in Fig. 3. The activity remains fairly constant to an SDS concentration of 0.8 μmole of SDS per mg of membrane protein, then drops off at higher concentrations. Triton X-100 caused some inactivation of the enzyme at all concentrations tested, exhibiting a much stronger inhibitory effect than SDS. The effects of the two detergents were dependent on the incubation medium. Ghosts incubated in Krebs-Ringer phosphate buffer appear to be completely rescaled (Fig. 4). The comparisons between the two detergents are somewhat surprising, since Triton X-100 is usually considered to be the milder detergent in terms of protein denaturation and is often used to solubilize membranes for enzyme assays (3). In view of the results further measurements of enzyme activities were performed after incubation in SDS in distilled water or other media of low ionic strength. The activities of aldolase, acetylcholinesterase, and the other enzymes assayed were inhibited only slightly or not at all under these conditions. The present results also indicate the care which must be taken in the use of detergents to study membrane-associated enzymes.

Effect of Trypsin Treatment on Association of Glyceraldehyde 3-Phosphate Dehydrogenase with Erythrocyte Membranes—Previous experiments by Steck et al. (6) and from our laboratory (4) have
shown that the polypeptide corresponding to the subunit of glyceraldehyde 3-phosphate dehydrogenase is very resistant to proteolysis by trypsin or chymotrypsin. This can be demonstrated by either staining of SDS acrylamide gels or iodoacetate labeling. Steck et al. (6) have also noted that this protein was released from the ghost during proteolysis. In order to examine the association of the dehydrogenase with the erythrocyte membrane, the proteolysis effects were investigated further. Isolated human erythrocyte membranes were treated with trypsin and centrifuged. The pellet and supernatant samples were assayed for glyceraldehyde 3-phosphate dehydrogenase and showed a time-dependent release of the enzyme (Fig. 5). However, subsequent investigations showed several discrepancies with this type of experiment. First, the release of enzyme was highly variable between different membrane preparations. Second, assays of the trypsin-treated whole ghost samples (before centrifugation) showed a dramatic decrease of dehydrogenase activity, which was roughly inversely correlated with the release of the enzyme. Since the enzyme had not been removed from the suspension in this case or proteolytically cleaved, the loss of activity must be due to an effect at the membrane level rather than on the enzyme. Table II illustrates the effect of trypsin treatment on the glyceraldehyde 3-phosphate dehydrogenase and aldolase activities of the membrane fractions. In this experiment the treated ghost sample shows a loss of more than 50% of its glyceraldehyde 3-phosphate dehydrogenase activity before centrifugation. Both enzymes are released into the supernatant in the treated samples but not in the controls.

The most logical explanation for the results of Table II is that the ghosts reseal upon trypsin treatment and thus show an apparent loss of enzyme activity. In order to test this proposal trypsin-treated and control membranes were assayed in the presence of SDS. The membranes were also centrifuged to yield a supernatant and pellet fractions. This pellet (Pellet I) was assayed in the presence of SDS or subjected to a freeze-thaw procedure. The frozen-thawed membranes were also centrifuged to yield a supernatant and pellet fractions which were assayed in the presence or absence of SDS. The results of these determinations are shown in Table III and are consistent with the resealing hypothesis. Trypsin treatment of the ghosts causes an apparent loss of activity that is not observed if the samples are incubated with detergent (Column 1 versus 2). Almost no enzyme was released upon trypsin treatment and thus show an apparent loss of enzyme activity.

![Fig. 5](left). Trypsin release of glyceraldehyde 3-phosphate dehydrogenase from erythrocyte membranes. Ghosts (2.7 mg of protein per ml) were incubated at room temperature with trypsin (10 μg per ml) in 7 mM phosphate (pH 7.4). At timed intervals soybean trypsin inhibitor was added to a concentration of 20 μg per ml and the mixture was centrifuged. Supernatant and pellet samples were assayed for glyceraldehyde 3-phosphate dehydrogenase without detergent incubation. Supernatant (○---○) and pellet (●---●).

![Fig. 6](center). Release of glyceraldehyde 3-phosphate dehydrogenase at low trypsin concentration. Ghosts (2 mg of protein per ml) were incubated at room temperature with trypsin (1 μg per ml) and trypsin (10 μg per ml) in 7 mM phosphate (pH 7.4). Aliquots for centrifugation were treated with a 10-fold excess of soybean trypsin inhibitor. Supernatants were assayed for dehydrogenase. Aliquots of the reaction mixture were also solubilized directly in SDS for electrophoresis.

![Fig. 7](right). SDS acrylamide electrophoresis of trypsin-treated membranes. Conditions for trypsinization are given in Fig. 6. Gels A and B, controls; Gel C, 5 min; Gel D, 10 min; Gel E, 20 min; and Gel F, 40 min.

## Table II

<table>
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<th>Sample</th>
<th>Glyceraldehyde 3-phosphate dehydrogenase</th>
<th>Aldolase</th>
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<td></td>
<td>Control</td>
<td>Trypsin</td>
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<tr>
<td>Whole ghost</td>
<td>19.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Supernatant</td>
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leased into the supernatant in the trypsin treatment (Column 3), and the activity in the pellet is virtually all in a cryptic form, i.e., inaccessible to substrate (Column 4 versus 5). The 20% loss of activity in the treated pellet is unexplained. Perhaps release of the enzyme inside the ghost may subject it to other modes of inactivation. This enzyme is rather susceptible to oxidative inactivation. Freezing and thawing the pellet fraction results in a partial release of dehydrogenase from the treated sample but not the control sample (Column 6). The activity of the enzyme in the pellet from the freeze-thaw procedure is still in the cryptic form for the treated sample (Column 7 versus 8), suggesting that the freeze-thaw treatment has not opened these vesicles completely. The control sample shows no cryptic activity at any stage, although it, as well as the treated sample, has lost 40% of its activity after freezing and thawing.

To gain some information about the proteins destroyed during the process of glyceraldehyde 3-phosphate dehydrogenase release, it was necessary to observe protein patterns on a membrane sample from which a significant quantity of the enzyme was released. Figs. 6 and 7 show the enzyme release and SDS acrylamide gel protein patterns, respectively, for membranes treated at a trypsin concentration low enough to prevent extensive protein degradation. A maximum of about 25% of the dehydrogenase activity was released into the supernatant during the digestion. This probably represents only part of the enzyme released from the membranes, since a portion of that released from the membrane is probably trapped within the sealed ghosts. The most interesting observation of this experiment is that the release of the dehydrogenase reaches a maximum value without significant proteolysis of Component I (spectrin) of the membrane (Fig. 7). Greater than 85% of the staining density of spectrin was shown to be present by gel scans after a 20-min proteolysis. Since the spectrin in this region is unaltered by proteolysis, the proportionality between protein staining and protein amount remains constant. These results suggest that the dehydrogenase is not bound to the spectrin, although proteolysis of other membrane components might affect the enzyme-spectrin interaction and cause release of the dehydrogenase. These observations are pertinent in the light of previous reports of the binding of aldolase and glyceraldehyde 3-phosphate dehydrogenase to actin (23), which is quite similar to spectrin in many of its properties (24). That glyceraldehyde 3-phosphate dehydrogenase is not bound to spectrin is also suggested by the fact the spectrin can be almost completely extracted from the erythrocyte membrane without removal of the dehydrogenase (25, 26).

Effect of ATP and Other Membrane-perturbing Agents on Binding of Glyceraldehyde 3-Phosphate Dehydrogenase to Erythrocyte Membrane—Our early experiments with iodoacetate were designed to use this reagent as a probe for membrane changes resulting from addition of membrane effector molecules such as ATP or divalent cations. It is known that Ca++ causes loss of deformability and shrinkage of the resealed ghost when introduced during hemolysis (27). ATP causes an apparent conformational change of proteins of the isolated erythrocyte membrane to yield more β structure, as determined by infrared spectroscopy (28). Experiments were therefore conducted in which erythrocytes were hemolyzed in the presence of low concentrations of effectors, ressealed by addition of salt, labeled with radioactive iodoacetate, rehemolyzed, and washed to obtain hemoglobin-free ghosts. In these experiments a double labeling procedure was used (29). The control (no effector) was labeled with [4C]iodoacetate, and the effector-treated sample was labeled with [3H]iodoacetate. Under the labeling conditions used only two membrane proteins were significantly labeled, Component I (spectrin) and Component VIII (glyceraldehyde 3-phosphate dehydrogenase). Only one of the four effectors tested (Mg++, Ca++, ATP, and EDTA) caused a significant alteration in the labeling pattern. ATP greatly decreased the labeling of the second band (glyceraldehyde 3-phosphate dehydrogenase) as shown in Fig. 8. Two possible explanations for this decrease can be postulated. (a) There was a loss of the enzyme from the membrane as a result of the ATP treatment, or (b) there was a direct effect of ATP on the enzyme. By scanning SDS acrylamide gels of the control and treated samples, it was possible to show that the former proposition was correct. The identification of the labeled component as glyceralde-

**Table III**

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![Fig. 8. Double labeling of resealed human erythrocyte ghosts prepared in the presence and absence of ATP. Two batches of released ghosts were prepared as described previously (4), except in one batch ATP (2 mM) was included in the hemolysis mixture. After rescaling, the ghost prepared in the presence of ATP were treated with [3H]iodoacetate and those prepared in absence of ATP were treated with [3C]iodoacetate. The membranes were washed with hypotonic phosphate to remove hemoglobin. The two membrane samples were combined, solubilized in SDS, and subjected to SDS acrylamide electrophoresis as previously described (4). The protein pattern by Coomassie blue staining did not differ significantly from that of membranes not treated with iodoacetate. Samples of 4,700 and 40,000 dpm of 3C and 3H, respectively, were applied to the gels. Fifty per cent of the two labels was recovered from the gels. No attempts were made to increase recoveries for these particular experiments, 3C, ---; 3H, --.](http://www.jbc.org/content/144/1/1414/F12)
hyde 3-phosphate dehydrogenase then indicated that the binding of this key glycolytic enzyme to the erythrocyte membrane was influenced by the presence of ATP.

Table IV shows the release of glyceraldehyde 3-phosphate dehydrogenase, lactic dehydrogenase, and hemoglobin into the supernatant during hemolysis in the presence of various effector substances. The activities are expressed as a percentage of the total activity (pellet plus supernatant). Pellet activities were measured in the presence of detergent. The values in Table IV were not corrected to include the amount of soluble enzyme in the pellet fraction. All subsequent values have been corrected. The effects of various substances added on the release of glyceraldehyde 3-phosphate dehydrogenase activity are consistent with the results of the earlier labeling study. Only ATP causes significantly enhanced release. The other substances are relatively ineffective at the concentrations used. The presence of Mg\(^{2+}\) inhibits the ATP effect. Lactic dehydrogenase and hemoglobin release are essentially complete, if correction is made for the volume of the pellet. ATP has no effect on these, but divalent cations slightly enhance retention on the membrane. In a number of experiments of this type the amount of glyceraldehyde 3-phosphate dehydrogenase associated with the erythrocyte membrane was 60 to 80\% after hemolysis in Tris buffer. If ATP was present in the hemolyzing medium, this value dropped to 5 to 20\%.

**Table IV**

<table>
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<th>Substance released</th>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>45</td>
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<tr>
<td>Lactic dehydrogenase</td>
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<td>87</td>
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<td>Hemoglobin.........</td>
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Fig. 9. Concentration dependence of ATP and Ca\(^{2+}\) effects on the release of glyceraldehyde 3-phosphate dehydrogenase during hemolysis. Hemolysis conditions are given under "Experimental Procedures." Values in this experiment were not corrected for soluble enzyme in the pellet fraction. Total activity values (100\%) were 13.8 and 32.1 \(\mu\)moles per min per ml of erythrocytes for glyceraldehyde 3-phosphate dehydrogenase and lactic dehydrogenase, respectively. Hemoglobin value was 294 \(\mu\)g per ml of erythrocytes.

ATP effect on glyceraldehyde 3-phosphate dehydrogenase release at 0\°. It was also shown that Ca\(^{2+}\) can cause a release of the enzyme, although it requires a higher concentration than ATP. The concentration dependence of the release of the enzyme by 2,3-diphosphoglycerate was quite similar to that observed with Ca\(^{2+}\) (data not shown).

The effects of ATP on release of erythrocyte glyceraldehyde 3-phosphate dehydrogenase from the membrane were of particular interest in view of the previously demonstrated effects of ATP on the soluble rabbit muscle and yeast enzymes (8–10). To determine if similar behavior is exhibited by the erythrocyte enzyme, the solubilized enzyme was treated under conditions similar to those used previously. As shown in Table V, the erythrocyte enzyme is also inhibited by ATP at 0\° but not at 25\°. The inhibition can be prevented by preincubation with NAD, but it is only partially reversed if NAD is added after incubation with ATP. In addition ATP has been shown to destabilize the enzyme toward proteolytic inactivation with chymotrypsin (Fig. 10). Again, NAD preincubation prevents this ATP effect. To determine if the effects of ATP on the soluble enzyme are correlated with the release of the enzyme from the membrane,

**Table V**

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<th>EFFECTOR CONCENTRATION (mM)</th>
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<th>0°</th>
<th>0°</th>
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</table>

* RT, room temperature.

**Fig. 10.** Effect of ATP and NAD on chymotrypsin inactivation of glyceraldehyde 3-phosphate dehydrogenase. Incubation conditions are given under "Experimental Procedures." Activities are expressed as percentage of initial activity for each sample. Control (○—○), plus ATP (●—●), and plus ATP and NAD (▲—▲). Control activity (100\%) was 2.9 \(\mu\)moles per min per mg of protein.
two different experiments were performed. In the first it was shown that NAD effects a somewhat enhanced release of the dehydrogenase in the absence of ATP, but it does not inhibit the ATP-promoted release. In addition it was shown that hemolysis at 25°C in the presence of ATP showed the same ATP concentration dependence of the glyceraldehyde 3-phosphate dehydrogenase release as hemolysis at 0°C. Thus there does not appear to be any direct correlation between the inactivation or conformation changes of the enzyme and the release of the enzyme during hemolysis. The enzyme release is not dependent on either temperature or NAD preincubation, while the inactivation is dependent on both.

**Discussion**

The glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase is only partially released from the erythrocyte during hypotonic hemolysis; the bulk of the enzyme is usually associated with the membrane. Release can be effected by treatment of the isolated membranes with salt solutions at moderately high ionic strengths. This effect suggests ionic interactions as the primary mode of binding the enzyme to the membrane. Such a proposition must be advanced cautiously, since it is known that increased ionic strengths cause significant changes in erythrocyte membrane structural parameters (30). A salt effect on the membrane could alter the membrane-enzyme association in the absence of specific ionic interactions between the two. The converse argument, i.e. that the failure to release a membrane-bound protein by high salt extraction is evidence against ionic association, must also be considered suspect in some cases. For example, the erythrocyte membrane protein spectrin is released from the membrane at low ionic strengths and high pH in the presence of EDTA, suggesting that its association with the membrane may involve ionic interaction. However, spectrin is not released at high ionic strengths (26) because it aggregates and becomes insoluble under these conditions. Thus a protein ionically bound to the membrane might not be detected by salt treatment. Three major components, spectrin (Component I), glyceraldehyde-3-phosphate dehydrogenase (Component VIII), and Component VII, representing 30 to 50% of the membrane protein, can be released in disaggregated form by mild procedures which do not destroy the membrane structure, although they do fragment the membranes. All of these can thus be considered peripheral proteins by most definitions. The observation by Reynolds and Trayer (21) that 90% of the erythrocyte membrane protein can be solubilized by 5 mM EDTA suggests an even higher proportion of peripheral protein. However, it was not demonstrated in this case that all of this protein was disaggregated from lipids or from other proteins. The ability of erythrocyte ghosts to seal must be considered in any studies on the structure of the isolated membranes. This sealing has been monitored in our laboratory by glyceraldehyde 3-phosphate dehydrogenase activity and protolysis, but measurement of the exclusion of membrane-impermeable molecules can also be used (7, 32). Considerable variability in the ability to reseal has been noted between different membrane preparations. Aging at low temperature and freezing and thawing both reduce the ability of ghosts to reseal. It was interesting to note that trypsinization of the ghosts effected resealing. The proportion sealed by trypsin is also dependent on prior treatment. The process is quite rapid, which suggests that cleavage of a limited number of polypeptides may be involved. Component IVa of the erythrocyte membrane is the most rapidly cleaved of the major erythrocyte polypeptides (7), but there is no direct evidence that this cleavage is involved in resealing. Resealing of the ghost does not prevent further digestion of the membrane polypeptides, indicating that trypsin is present on both sides of the membrane before resealing. Digestion cannot be halted completely by addition of protein inhibitors of the protease, since the protease on the inside is inaccessible to external proteins. Whether these observations might be pertinent to other membrane types is unknown, but appropriate cautions should be exercised in interpreting studies on protease digestion of membranes of all types.

By analogy with previous work on the rabbit muscle and yeast enzymes (8-10), it appears that in the presence of ATP the erythrocyte glyceraldehyde 3-phosphate dehydrogenase undergoes a conformational change which leads to an eventual dissociation into subunits at low temperatures. These changes are apparently not involved in the release of the dehydrogenase from the membrane, since the ATP-promoted release is not temperature-dependent and is not affected by NAD. The ATP effect on the enzyme release may result from a direct effect on the erythrocyte membrane. Graham and Wallach (28) have shown evidence of ATP-induced conformational changes on erythrocyte membrane proteins by infrared spectroscopy. Calcium can also alter membrane structure, as indicated by fluorescent probes (30) and by Ca++-induced proteolysis and aggregation of erythrocyte membrane proteins (31). This latter effect occurs over the same range of Ca++ concentrations which cause the dehydrogenase release.

The difference between the effect of ATP on the inactivation of the soluble enzyme and on enzyme release is not too unexpected, since the concentration of free ATP which is available for binding to the dehydrogenase is probably not very great in the hemolysis system because of the ATP-binding sites on the membrane or hemoglobin.

The physiological significance of the ATP-promoted release of the dehydrogenase from the membrane is still a matter for conjecture. Any attempt to answer this question must first consider whether the dehydrogenase (and other glycolytic enzymes) is bound to the membrane in the intact cell or whether it becomes associated during hemolysis. One can envision four different possibilities for the arrangement of the dehydrogenase and other glycolytic enzymes of the intact red cell: (a) the enzymes are all soluble in the cell, (b) some of the enzymes are soluble and some are membrane-bound, (c) all of the enzymes are bound to the membrane, or (d) the enzymes are situated between the interior surface of the membrane and the fibrous network at or near the interior surface. The last organization would be similar to the periplasmic space of gram-negative bacteria. In this case the bulk of the enzymes would be released by hypotonic shock (hemolysis), which could partially free the fibrous material from its association with the membrane. Only those enzymes most closely associated with the membrane would not be released. This type of explanation might account for the large variations noted in the tendencies of different erythrocyte enzymes to associate with the membrane during hemolysis (3). In any of the cases in which the enzymes are bound directly to the membrane, there is a potential for some control of the enzyme activities by substances which act on the membrane. In the last example an additional perturbation of activities might be achieved through the fibrous protein. Experimental results are currently too limited to suggest particular examples of this type of control, but such mechanisms should be considered along with substrate levels and allosteric effects as a mode of controlling metabolic fluxes. Further studies are obviously needed to determine the states of
enzymes in intact cells and how these states may affect their functions.

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