An ATP-modulated Specific Association of Glyceraldehyde-3-phosphate Dehydrogenase with Human Erythrocyte Glucose Transporter*

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Glyceraldehyde-3-phosphate dehydrogenase was found to bind in vitro to purified, human erythrocyte glucose transporter reconstituted into vesicles. Mild trypsinic digestion of the glucose transporter totally inactivated the binding, suggesting that the cytoplasmic domain of the transporter is involved in the binding to glyceraldehyde-3-phosphate dehydrogenase. The binding was abolished in the presence of antisera raised against the purified glucose transporter, further supporting the specificity of this interaction. The binding was reversible with a dissociation constant (K_a) of 3.3 x 10^{-6} M and a total capacity (B_max) of approximately 30 nmol/mg of protein indicating a stoichiometry of one enzyme-tetramer per accessible transporter. The binding was sensitive to changes in pH showing an optimum at around pH 7.0. KCl and NaCl inhibited the binding in a simple dose-dependent manner with K_i of 40 and 20 mM, respectively. The binding was also inhibited by NAD^+ with an estimated K_i of 3 mM. ATP, on the other hand, enhanced the binding by up to 3-fold in a dose-dependent manner with an apparent K_i of approximately 6 mM. The binding was not affected by D-glucose or cytochalasin B. The binding did not affect either the glucose or cytochalasin B in binding affinities or the transport activity of the transporter. However, the enzyme was inactivated totally upon binding to the transporter. Based on these findings, we suggest that a significant portion of glyceraldehyde-3-phosphate dehydrogenase in human erythrocytes exists as an inactive form via an ATP-dependent, reversible association with glucose transporter, and that this association may exert regulatory intervention on nucleotide metabolism in vitro.

Utilization of glucose in animal cells starts with its stereospecific uptake across the plasma membrane diffusion barrier catalyzed by a specific membrane protein (transporter) (1). Recent molecular cloning studies have disclosed several tissue-specific glucose transporter isoforms operative in different cells such as human erythrocytes, brain, liver, muscle, and fat cells (2–6). Of these, the human erythrocyte glucose transporter (HEGT) is the only isoform that has been purified in a large quantity (7). Consequently, a significant amount of biochemical and structural information is available for this isoform (8–12). This information on HEGT, together with the cDNA-deduced primary structure of this and other isoforms, indicates that all glucose transporter isoforms show a common structural motif, possessing a transmembrane domain made of 12 membrane-spanning a-helices, a large cytoplasmic domain, and a relatively small carbohydrate-containing exoplasmic domain. The transmembrane domain is well conserved among different isoforms and is suggested to form an aqueous pore for substrate passage (2, 9) common to all isoforms. On the other hand, a significant degree of tissue specificity was found in the primary structure of both the cytoplasmic and exoplasmic domains of these isoforms, suggesting that these domains may play an important role in tissue-specific regulation of glucose transport function and cellular glucose utilization, via specific interaction with certain cytosolic proteins.

In adipocytes and muscle cells, the overall rate of glucose utilization is regulated at the transport step by insulin (1, 13). Insulin stimulates glucose transport in these cells largely by redistributing glucose transporters from an intracellular reserve pool to the plasma membrane (13). Although the exact mechanism is not known, it is likely that this insulin-induced transporter redistribution may involve direct molecular interaction of a specific cellular protein or proteins with the cytoplasmic domain of the glucose transporter. A number of phosphoproteins whose phosphorylations were shown to be insulin-dependent (14, 15) may play a role in this putative interaction.

In other cells such as human erythrocytes, hepatocytes, and brain cells, the rate of glucose transport is much faster than the rate of overall glucose utilization (1). Glucose utilization in these cells is regulated at specific individual enzyme steps rather than at the transport step. For the glycolytic pathway in human erythrocytes, hexokinase, phosphofructokinase, and pyruvate kinase are shown to be rate-limiting steps (16). However, it is still possible that the glucose transporter may interact with these rate-limiting enzymes and thus may control glucose utilization indirectly. Specific interaction of certain glycolytic enzymes with the anion transporter in human erythrocytes is well known (17, 18). Furthermore, it has been suggested that many enzymes in the glycolytic pathway form multimolecular complexes and regulate their own activities (19).

* This work was supported in part by National Institutes of Health Research Grant DK-13376, American Heart Association, and the Veterans Administration Medical Center, Buffalo, NY. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: HEGT, human erythrocyte glucose transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NaDodSO4, sodium dodecyl sulfate; MOPS, 4-morpholinopropanesulfonic acid.
The present study was undertaken to determine if such a putative interaction of cytosolic protein(s) with glucose transporter occurs in human erythrocytes. When purified human erythrocyte glucose transporter vesicles were incubated with human erythrocyte hemolysate, several cytosolic proteins were found to be associated with the glucose transporter with high affinities. One of them was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Partial characterization of this binding suggests that a significant portion of this enzyme may exist in association with the glucose transporter in vivo. We demonstrate that this association requires an intact cytoplasmic domain of the transporter. We also demonstrate that ATP stimulates this association and the association inhibits the enzyme activity. We propose that the glucose transporter may exert regulatory intervention on nucleotide metabolism via an ATP-regulated specific association with GAPDH in human erythrocytes.

EXPERIMENTAL PROCEDURES

Materials—Oxidized human whole blood was supplied by the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. Cytochalasin B was obtained from the American Red Cross, Buffalo, NY. 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Alicquot of HEGT-vesicles fold diluted hemolysate in 500 ~1 of 1:15 isotonic phosphate buffer for 30 min, the mixture was centrifuged (300,000 × g, 120 min), and the resultant pellet was analyzed on 10% acrylamide as described under "Experimental Procedures." Panel A, HEGT-vesicles after incubation with hemolysate at varying pH; lanes 1 to 6 are pH 4.0, 5.6, 6.4, 7.0, 8.1, and 8.9, respectively. Lanes 7 and 8 are molecular markers, whose molecular weight values (x10^2) are shown in the right margin. Arrowhead indicates the M, = 36,000 polypeptide position. Panel B, HEGT-vesicles were incubated without (lane 1) or with (lanes 2–5), 0.3 μM GAPDH in 500 μl of 1:15 isotonic phosphate buffer, pH 7.4, in the absence (lanes 1 and 5) or in the presence of nonimmune antisera (lane 2) or immune antisera A4-1 or 60 (lane 3) or 120 (lane 4) μg of protein equivalent. The relative bond intensities of GAPDH based on densitometry were 1.00 (lane 2), 0.72 (lane 3), 0.51 (lane 4), and 0.93 (lane 5), respectively.

Fig. 1. NaDodSO4-polyacrylamide gel electrophoreses of human erythrocyte hemolysate proteins and purified human erythrocyte GAPDH co-precipitated with HEGT-vesicles. An aliquot of HEGT-vesicles (40 μg of protein) was incubated with 3-fold diluted hemolysate in 500 μl of 1:15 isotonic phosphate buffer for 30 min, the mixture was centrifuged (300,000 × g, 120 min), and the resultant pellet was analyzed on 10% acrylamide as described under "Experimental Procedures." Panel A, HEGT-vesicles after incubation with hemolysate at varying pH; lanes 1 to 6 are pH 4.0, 5.6, 6.4, 7.0, 8.1, and 8.9, respectively. Lanes 7 and 8 are molecular markers, whose molecular weight values (x10^2) are shown in the right margin. Arrowhead indicates the M, = 36,000 polypeptide position. Panel B, HEGT-vesicles were incubated without (lane 1) or with (lanes 2–5), 0.3 μM GAPDH in 500 μl of 1:15 isotonic phosphate buffer, pH 7.4, in the absence (lanes 1 and 5) or in the presence of nonimmune antisera (lane 2) or immune antisera A4-1 or 60 (lane 3) or 120 (lane 4) μg of protein equivalent. The relative bond intensities of GAPDH based on densitometry were 1.00 (lane 2), 0.72 (lane 3), 0.51 (lane 4), and 0.93 (lane 5), respectively.

ity identical with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (17), suggesting identity. Based on this suggested identity, we investigated the interaction of partially purified human erythrocyte GAPDH with HEGT-vesicles. HEGT-vesicles were incubated in 1:15 isotonic phosphate buffer with GAPDH for 30 min, pelleted by centrifugation, and recovered free of incubation medium. Gel electrophoresis of the pelleted vesicles showed an additional 36,000-Da peptide band (Fig 1B), demonstrating that GAPDH indeed binds to the HEGT-vesicles. The binding increased with an increasing concentration of GAPDH in saturable manner (Fig. 2). In control experiments, where protein-free lipid vesicles prepared from a total lipid extract of human erythrocyte membranes were used rather than HEGT-vesicles, there was negligible binding of the enzyme to the vesicles (Fig. 2), indicating that the binding is specific to protein, but not to lipid. More than 50% of the GAPDH binding to HEGT-vesicles was inhibited in the presence of antisera A4-1, and this inhibition was dose-dependent (Fig 1B). A4-1 was known to interact with HEGT at its cytoplasmic domain (not illustrated), further indicating that the enzyme binding is specific to HECT.

HEGT in the HEGT-vesicles used here exist in random transmembrane orientation of approximately 45% right-side-out and 55% inside-out dispositions (data not illustrated). When HEGT-vesicles were treated with trypsin under controlled conditions (10, 25) and subjected to NaDodSO4-gel electrophoresis, 52–55% of the HEGT were found to be digested into 23,000- and 19,000-Da fragments (data not illustrated). This indicated that practically all the HEGT existing in the inside-out transmembrane orientation in vesicles were digested. Such digestion also removed the cytoplasmic hydrophilic domains of HEGT including the central loop and both the amino and carboxyl termini (10). If the HEGT-vesicles thus digested were incubated with GAPDH under otherwise identical conditions, there was little protein-specific enzyme binding to the HEGT-vesicles (Fig. 2). This finding demonstrates that the enzyme binds to the glucose transporter at its cytoplasmic domain. This is consistent with the observation that GAPDH does not bind to intact cell or resealed ghosts of human erythrocytes in suspension (17).

Protein-specific, GAPDH binding was estimated from the difference in the binding to HEGT-vesicles over that bound to the corresponding amounts of protein-free lipid vesicles (Fig. 2). A double reciprocal plot of the protein-specific binding (Fig. 3) revealed that the binding is indeed a saturable function of enzyme concentrations. It showed a single binding component with an apparent dissociation constant (Kd) of 3.3 ± 1.1 x 10^-6 M and total capacity (Bmax) of 31 ± 3.2 nmol of GAPDH monomers/mg of HEGT-vesicle protein (means ± S. D. from six measurements). Since the HEGT-vesicles used here bound cytochalasin B with specific activities of 15 ± 1.2 nmol/mg of protein, and approximately 55% of the HEGT in these preparations were in an inside-out transmembrane orientation, the observed binding capacity corresponds to a binding stoichiometry of 4 GAPDH monomers for each HEGT monomer or one-to-one binding of the enzyme to the transporter.

Fig. 4 shows the binding of GAPDH to HEGT-vesicles as a function of pH in the incubation mixture. It clearly shows that there is a pH optimum for the binding and it occurs at a...
against that of pH 7.4. Inset, Coomassie Blue stain intensities of GAPDH in 1:15 isotonic phosphate buffer in a final volume of 0.5 ml. The enzyme binding at different pH values were normalized against that of pH 7.43. Inset, gel electrophoresis data of GAPDH binding at pH (from right to left) 5.4, 6.54, 7.43, 8.49, 9.42, and 10.2, respectively. Arrowhead indicates GAPDH bound.

Fig. 4. Effects of pH on GAPDH binding to HEGT. Binding assay mixture contained HEGT-vesicles (45 μg of protein) and 0.3 μM GAPDH in 1:15 isotonic phosphate buffer in a final volume of 0.5 ml. The enzyme binding at different pH values were normalized against that of pH 7.43. Inset, gel electrophoresis data of GAPDH binding at pH (from right to left) 5.4, 6.54, 7.43, 8.49, 9.42, and 10.2, respectively. Arrowhead indicates GAPDH bound.

Fig. 5. Effects of KCl and NaCl on GAPDH binding to HEGT as a function of ion concentrations. HEGT-vesicles (48 μg of protein) were incubated with 0.3 μM (●, △) or 1.0 μM (○) GAPDH in 1:15 isotonic phosphate buffer (pH 7.4) supplemented with a given (on abscissa) concentration of KCl (●) or NaCl (△, ○). The binding was quantitated as described in Fig. 2 legend. Inset, gel electrophoresis data for NaCl with 0.3 μM GAPDH. Arrowhead indicates GAPDH position. NaCl concentrations were (from 2nd left to right) 0, 10, 30, 60, 120, 150 mM, respectively. Molecular markers were shown in the leftmost lane. Quantitation of binding was done as in Fig. 2.

pH between 6.5 and 7.5. The binding was greatly reduced at either side of this optimum pH, generally reproducing the pH effect seen with hemolysate (Fig. 1).

The data illustrated in Fig. 5 show that the enzyme binding to HEGT-vesicles is also sensitive to changes in ionic strength. Either KCl or NaCl, added to the incubation mixture at an increasing concentration, reduced the binding in a dose-dependent manner. When HEGT-vesicles containing 45 μg of protein were incubated with 0.3 μM GAPDH, as much as 50% of the binding was inhibited. Under these conditions, the nonspecific binding of GAPDH to lipid amounted to almost 50% of the total binding to the vesicles (Fig. 2), indicating that the entire protein-specific binding was affected. Scatchard analyses (not illustrated) revealed an apparent inhibition constant (K<sub>i</sub>) of approximately 40 and 25 mM for KCl and NaCl, respectively. The K<sub>i</sub> value for KCl was not affected when GAPDH concentration was increased from 0.3 to 1.0 μM (Fig. 5) indicating that the inhibition is noncompetitive.

In efforts to further assess possible physiological relevance of the GAPDH binding to HEGT-vesicles observed here, a number of intracellular metabolites were tested if they affect the binding. NAD<sup>+</sup> inhibited the binding effectively with a clear dose dependence (Fig. 6). The entire protein-specific binding was inhibited by this metabolite, with a half-maximum inhibition at approximately 3 mM NAD<sup>+</sup>. 3-Phosphoglycerate, ADP, and AMP did not produce any significant effect on the binding (not illustrated). ATP, on the other hand, was found to enhance the enzyme binding to HEGT-vesicles (Fig. 6). The ATP effect showed a complex dose dependence. There was little effect or slight reduction in binding at ATP concentrations of 2 mM or lower. This was followed by a steep increase between 3 and 10 mM ATP, then gradually approaching an apparent maximal level of at least a 3-fold increase obtained at 20 mM ATP. ATP is known to dissociate the enzyme tetramer into monomeric forms. If the tetrameric form is required for the enzyme binding to the transporter as is suggested by the observed binding stoichiometry, the effect of ATP on the enzyme binding to HEGT calculated here most likely represents an underestimation. The half-maximum of the ATP effect was achieved at an ATP concentration of approximately 6 mM.

A typical transporter substrate, D-glucose (up to 500 mM), and a specific inhibitor of the transporter, cytochalasin B (as high as 10<sup>-5</sup> M), did not affect the specific association of GAPDH to HEGT-vesicles (not illustrated). We next examined whether the enzyme binding affects the transporter activities. Incubation of HEGT-vesicles with GAPDH at a concentration as high as 20 μM, the in vitro condition when close to 50% of the total HEGT would be expected to form a complex with GAPDH, did not produce any detectable effect on cytochalasin B binding activity of HEGT (not illustrated). D-Glucose displaced cytochalasin B binding from HEGT-vesicles equally well in the presence of 6 μM GAPDH as it did in the absence of GAPDH (Fig. 7). Furthermore, incubation of HEGT-vesicles with 6 μM GAPDH did not affect the transport activity of HEGT (Fig. 8). More than 70% of accessible HEGT (or 35% of total HEGT) was expected to be complexed with GAPDH at this concentration. These results clearly demonstrate that the specific association of GAPDH does not affect HEGT either in its inhibitor and substrate binding activities or its transport activity.

When incubated with HEGT-vesicles, GAPDH was found to lose its enzymatic activity significantly (Fig. 9). This reduction in GAPDH enzyme activity was found to directly correlate with the amounts of the enzyme bound to the transporter. Analysis of this correlation demonstrated that the binding results in total inactivation of the enzyme.
An aliquot of 890 ng of GAPDH in Tris (100 mM)-HCl buffer in a final volume of 1.0 ml was incubated without (O) or with (0) HEGT-vesicles (44 ng of protein) at 21 °C for 30 min before initiation of the enzyme-catalyzed reaction (see "Experimental Procedures"). 80 to 85% of GAPDH was estimated to be in complex with HEGT under the conditions employed.

**DISCUSSION**

We have demonstrated in the present study that GAPDH binds *in vitro* to purified glucose transporter vesicles of human erythrocytes. This binding is due to protein, rather than lipids of the vesicles, as protein-free lipid vesicles do not bind the enzyme with any measurable affinity (Fig. 2). More than 93% of the protein of the HEGT-vesicles used here is glucose transporter (20) and 2–5% of the protein is nucleoside transporter, indicating that the binding is essentially to HEGT. The binding is indeed specific to HEGT, as anti-HEGT immunsera, A4-1, effectively inhibited the enzyme binding (Fig. 1B). The binding requires intact cytoplasmic domain of the transporter, since cleaving this domain by trypsin totally abolished the binding (Fig. 2). The binding measured at pH 7.4 and a low (10 mM) ionic strength shows a *K*~d~ of 3.3 μM and a stoichiometry of one enzyme tetramer per transporter. The binding is sensitive to changes in pH and is maximal at near the physiological pH. The binding is inhibited by KCl or NaCl, with apparent inhibition constants of 40 and 25 mM, respectively. This ionic strength effect alone suggests that the binding would be minimal in intact cells.

The GAPDH binding to glucose transporter is inhibited by NAD⁺ and enhanced by ATP (Fig. 6). The NAD⁺ effect, however, required much (at least 100 times) higher concentrations than the physiological intracellular concentration of this metabolite (26). The enhancement of ATP, on the other hand, was effective at ATP concentrations between 2 and 20 mM. These are significantly higher than the physiological ATP concentration in human erythrocytes (2-3 μM), but similar to the physiological ATP concentrations found in some other cells including rat adipocytes (7 μM) and muscle cells (20 μM). The exact molecular mechanism of this ATP effect on the binding is not known. Carruthers and his group (27) have shown that ATP interacts with the glucose transporter and implicated this interaction in the asymmetry of glucose transport kinetics which is specific to intact human erythrocytes. However, this particular interaction showed an apparent affinity constant of 40–50 μM, thus is unrelated to the ATP effect on the GAPDH binding described here.

The binding of GAPDH to the glucose transporter shows a number of similarities with the binding of the enzyme to the anion transporter (band 3) of human erythrocytes (17, 18). Both are reversed by an increasing ionic strength, suggesting the importance of electrostatic interaction in the binding (17). Both involve the cytoplasmic domain of the transporter for the interaction (18). Both result in inactivation of the enzyme (28). However, they also show important differences. The enzyme binding to glucose transporter is most effective at a near physiological pH, whereas the binding to anion transporter is maximal at an alkaline pH clearly outside of the physiological range (17). ATP stimulates the GAPDH binding to glucose transporter, whereas it prevents the enzyme binding to anion transporter (17). The affinity for the interaction with glucose transporter is almost 30 times lower than the affinity for the interaction with anion transporter (*K*~d~ = 1 × 10⁻⁷ M).

Total cellular content of glucose transporter in human erythrocyte has been estimated to be 2–3 × 10⁶ monomers/cell (29, 30). This is practically identical with the total cellular content of GAPDH (3 × 10⁶ tetramers/cell) in human erythrocyte (31). The total cellular content of anion transporter (10⁶ monomers/cell) is significantly greater than either of these proteins (31). GAPDH binding to the anion transporter in human erythrocytes has been estimated to be as high as 60% of the total cellular GAPDH content *in situ* (32).

A recent immunofluorescence study unequivocally demonstrates that GAPDH in human erythrocytes is indeed largely associated *in situ* with the plasma membrane (18). How much...
of this GAPDH association to the membrane is due to the binding to glucose transporter and how much is due to the anion transporter is an interesting open question, which cannot be answered by the in vitro data alone. In rat and chick erythrocytes, most GAPDH was found to be in soluble form rather than membrane-associated (28, 33). This observation was correlated with the fact that the anion transporter isoforms in these cells lack a number of acidic residues at the cytoplasmic domain where GAPDH may bind through electrostatic force. It is equally interesting to note the fact that these cells have a negligible amount of glucose transporter compared with human erythrocytes (29).

Physiological significance of the GAPDH binding to the glucose transporter described here is yet to be determined. The enzyme binding did not affect the transporter including its substrate binding activity, cytochalasin B binding activity, and transport activity. However, ATP modulated the GAPDH binding to HEGT, and the binding activated the enzyme. GAPDH incorporates inorganic phosphate into organic metabolite glyceraldehyde 3-diphosphoglycerate while reducing NAD⁺ to NADH. Capacity of GAPDH is so high in human erythrocytes that even 90% inhibition of this enzyme does not affect the overall glucose utilization. It is thus highly unlikely that the enzyme binding to the transporter directly modulates the rate of glycolysis. On the other hand, the GAPDH binding to the transporter may exert a significant modulation on NAD⁺-NADH balance. NADH is the most important cofactor for methemoglobin reduction, and any reduction in NADH production may cause disbalance in this important function. Physiological importance of such a modulation should be directly and quantitatively evaluated in future studies.

The results of this present study also suggest that there may exist certain specific cytosolic protein(s) in cells other than erythrocytes which bind to the cytoplasmic domain of glucose transporter and may serve an important regulatory function. Purified HEGT-vesicles may be useful in identifying such putative cytosolic proteins provided that the binding involves a cytoplasmic domain common to all glucose transporters. Preliminary results obtained from our laboratory indeed suggests that such a cytosolic protein exists in rat adipocytes, which will be discussed elsewhere.

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