Regulation of GLUT1-Mediated Sugar Transport by an Antiport/Uniport Switch Mechanism†

Erin K. Cloherty, Deborah L. Diamond,‡ Karen S. Heard, and Anthony Carruthers*

Department of Biochemistry and Molecular Biology, Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, Massachusetts 01605

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ABSTRACT: Avian erythrocyte sugar transport is stimulated during anoxia and during exposure to inhibitors of oxidative phosphorylation. This stimulation results from catalytic desuppression of the cell surface glucose transporter GLUT1 [Diamond, D., & Carruthers, A. (1993) *J. Biol. Chem. 268*, 6437−6444]. The present study was undertaken to investigate the mechanisms of GLUT1 suppression/desuppression. Sugar uniport (sugar uptake or exit in the absence of sugar at the opposite side of the membrane) is absent in normoxic avian erythrocytes, but sugar antiport (sugar uptake coupled to sugar exit) is present. Exposure to cyanide and/or to FCCP (mitochondrial inhibitors) stimulates erythrocyte sugar uniport but not sugar antiport. $K_{m(app)}$ for 3-*O*-methylglucose uniport and antiport are unaffected by metabolic poisoning. $K_{i(app)}$ for inhibitions of 3-*O*-methylglucose uniport by cytochalasin B and forskolin (sugar export site ligands) are unaffected by progressive stimulation of sugar uniport. Cyanide and FCCP stimulation of 3-*O*-methylglucose uniport are associated with increased AMP-activated protein kinase activity. Purified human GLUT1 is not phosphorylated by exposure to cytosol extracted from poisoned avian erythrocytes. FCCP does not stimulate GLUT1-mediated 3-*O*-methylglucose uptake in K562 cells but does increase K562 AMP−activated protein kinase activity. FCCP stimulation of 3-*O*-methylglucose uniport in resealed erythrocyte ghosts requires cytosolic ATP and/or glutathione. The nonmetabolizable ATP analog AMP-PNP cannot be substituted for ATP in this action. These results are contrasted with allosteric regulation of human erythrocyte sugar transport and suggest that avian erythrocyte sugar transport suppression results from inhibition of carrier uniport function. Uniport suppression is not mediated by interaction with cytosolic molecular species that bind to the sugar export site. The antiport to uniport switch mechanism requires ATP hydrolysis, is associated with elevated AMP-activated kinase function, and, if triggered by this kinase, is mediated by factors absent in K562 cells and downstream from the kinase.

Protein-mediated sugar transport is virtually absent in avian erythrocytes undergoing aerobic metabolism. When oxidative phosphorylation is inhibited by anoxia or by cyanide, avian red cell sugar transport is stimulated by 50-fold or more (Diamond & Carruthers, 1993; Simons, 1983a; Wood & Morgan, 1969). Transport stimulation results from desuppression of cell surface glucose transport proteins (GLUT1[1]) which are present at the plasma membrane at all times but which are catalytically suppressed when cellular demand for glycolytic ATP is low (Diamond & Carruthers, 1993). Sugar transport stimulation by cyanide and hypoxia in rat epithelial clone 9 cells and rat myocardiocytes also results in large part from desuppression of cell surface GLUT1 (Fischer et al., 1995; Shetty et al., 1993). This regulatory mechanism contrasts with insulin stimulation of sugar transport in adipose and insulin and exercise stimulation of sugar transport in skeletal muscle where transport acceleration may be quantitatively accounted for by translocation of a specific, intracellular glucose transporter (GLUT4) to the plasma membrane (Czech & Buxton, 1993; Holman et al., 1994; Hudson et al., 1993; Pilch et al., 1993). These differences suggest that discrete signaling pathways and/or glucose transport proteins mediate metabolic and endocrine control of sugar transport.

Several cytosolic parameters have been suggested to signal altered cellular metabolic status to catalytically suppressed surface glucose transporters. These include Ca^{2+} (Clausen, 1979; Elbrink & Bihler, 1975), AMP/ADP/ATP levels (Carruthers et al., 1989; Carruthers & Helgerson, 1989; Randle & Smith, 1958), cellular redox status (Czech, 1977), altered membrane phospholipid metabolism (Czech, 1977), and activation of guanine nucleotide-binding/exchange proteins (Baker & Carruthers, 1983). However, sugar transport regulation in nucleated red cells appears not to be explained by any of these putative signaling mechanisms (Wood & Morgan, 1969). The physical basis of glucose transport protein catalytic suppression during normoxia is unknown but could be twofold. Substrate binding sites may be rendered inaccessible, and/or substrate translocation steps may be inhibited.

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* Author to whom correspondence should be addressed. Phone: 508 856-5570. Fax: 508 856-6882. E-mail: anthony.carruthers@ummed.edu.

‡ Current address: Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164-4660.

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[1] Abbreviations: GLUT1, human erythrocyte glucose transport protein; 3OMG, 3-*O*-methylglucose; AMP-PNP, adenylyl imidodiphosphate; CCB, cytochalasin B; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*−(trifluoromethoxy)phenylhydrazone; GSH, reduced glutathione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RBC, red blood cell; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane.

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The present study was undertaken to investigate the signals that control GLUT1 catalytic suppression/desuppression and to examine the kinetic basis of GLUT1 suppression. Our findings suggest that suppressed GLUT1 functions as an antiporter. In this exchange-only mode, the transporter is incapable of net sugar transport and catalyzes only the futile exchange or antiport of extracellular sugar for intracellular sugar (Stein, 1986). Desuppression of avian erythrocyte sugar transport results from antiporter conversion to uniporter. In the uniport mode, the transporter not only catalyzes exchange sugar transport but also mediates sugar movements in the absence of exchangeable sugar at the opposite side of the membrane (Stein, 1986). This antiport/uniport switch mechanism is associated with activation of a cellular AMP-activated kinase during inhibition of cellular oxidative phosphorylation.

MATERIALS AND METHODS

Materials. [^{3}H]Cytochalasin B, [^{3}H]forskolin, [^{14}C]-3-*O*-methylglucose, [^{3}H]-3-*O*-methylglucose, [^{14}C]-D-glucose, and [γ-^{32}P]-ATP were purchased from DuPont New England Nuclear. Rabbit antisera raised against a synthetic carboxyl-terminal peptide of GLUT1 (intracellular residues 480−492; C-Ab) were obtained from East Acres Biologicals. SAMS peptide (HMRSAMSGLHLVKRR) was synthesized by the University of Massachusetts Medical Center Peptide Synthesis Facility. Outdated human blood was obtained from the University of Massachusetts Medical Center Blood Bank. Reagents were purchased from Sigma Chemicals. Pigeons were obtained from the Palmetto Pigeon Plant (GA). K562 cells were obtained from the ATCC.

Solutions. Pigeon saline consisted of 15 mM NaCl, 120 mM KCl, 0.5 mM $MgCl_2$, 60 mM sucrose, and 5 mM HEPES at pH 7.4 (24 °C). Pigeon red cell lysis medium contained 10 mM Tris-HCl, 4 mM $MgCl_2$, ±0.5 mM EGTA (pH 7.4) (24 °C), and ± 200 μM PMSF. Stopper consisted of Pigeon saline plus 100 μM phloretin and was equilibrated to ice temperature prior to use. Saline consisted of 150 mM NaCl, 5 mM Tris-HCl, and 0.2 mM EDTA at pH 7.4. KCl medium consisted of 150 mM KCl, 5 mM Tris-HCl, and 0.2 mM EDTA at pH 7.4. Lysis medium contained 10 mM Tris-HCl and 2 mM EDTA at pH 7.4. Tris medium consisted of 50 mM Tris-HCl and 0.2 mM EGTA at pH 7.4. Kinase assay medium consisted of 40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM DTT, and 5 mM $MgCl_2$. Extraction medium consisted of 0.5% Triton X-100, 250 mM mannitol, 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM sodium pyrophosphate, and 100 μM PMSF at pH 7.8 and 25 °C.

Preparation of Avian Erythrocytes and Avian Erythrocyte Ghosts. Pigeon blood was collected in citrate and filtered through cheese cloth to remove debris. The suspension was centrifuged at 3440*g* for 10 min at 4 °C. The supernatant and buffy coat were removed by aspiration, and red cells were resuspended in 10 volumes of pigeon saline and centrifuged again for 10 min. The wash/centrifugation cycle was repeated an additional two to three times. By this time, white cells were absent by two criteria: the lack of a visible buffy coat and the absence of nonerythroid cells as judged by phase contrast microscopy.

Pigeon, resealed red cell ghosts were prepared by hypotonic lysis of red cells in 40 volumes of ice-cold pigeon red cell lysis medium for 10 min. Membranes were collected by centrifugation at 19800*g* at 4 °C (15 min), resuspended in pigeon saline, and incubated at 37 °C for 40 min, and resealed ghosts were collected by centrifugation (10000*g*) for 5 min at 4 °C.

Preparation of Human Red Cells and Resealed Erythrocyte Ghosts. Washed red cells and resealed red cell ghosts were prepared as described in Carruthers and Melchior (1983). Red cells were obtained from outdated blood by three centrifugation/wash cycles in KCl medium. Between each centrifugation/wash step, the buffy coat containing white cells and platelets was carefully aspirated. Red cells were incubated in 100 volumes of KCl medium (1 h at 24 °C) to deplete intracellular glucose levels, then collected by centrifugation, and stored on ice. Red cell ghosts were prepared by dispersing 1 volume of cells in 40 volumes of ice-cold lysis medium. Following 10 min on ice, the suspension was centrifuged, the supernatant containing cellular contents aspirated, and the red cell membrane pellet gently resuspended in 10 volumes of KCl medium. The suspension was warmed to 37 °C and incubated for 40 min at this temperature. The resulting resealed ghosts were collected by centrifugation (10 min at 15000*g*), suspended in KCl medium, and placed on ice until use. When ghosts were formed containing ATP and/or other nucleotides, the relevant molecular species were included in the KCl medium solution (pH adjusted to 7.4) used to reseal the ghosts.

Tissue Culture. Human K562 leukemic cells were maintained in a 37 °C humidified CO_2 incubator in RMPI 1640 medium supplemented with 10% FBS, 100 u/mL penicillin, 100 μg/mL streptomycin, and 20 mM L-glutamine.

Human Erythrocyte Glucose Transport Protein. Tetrameric GLUT1 was purified from erythrocyte membranes as described previously (Hebert & Carruthers, 1991, 1992).

Ligand Binding Studies. Equilibrium [^{3}H]cytochalasin B binding to purified GLUT1, to GLUT1 present in human erythrocyte membranes depleted of peripheral proteins, to erythrocyte ghosts, and to intact erythrocytes was determined as described previously (Hebert & Carruthers, 1992; Helgerson & Carruthers, 1987).

Net 3-O-Methylglucose Uptake Measurements. The procedure for sugar transport measurements in avian red cells was as described by Diamond and Carruthers (1993). Cells were resuspended to a final hematocrit of 20% in saline. Unlabeled and [^{14}C]-3-*O*-methylglucose were added at time zero, and uptake was allowed to proceed for 10 min at 37 °C. At this time, 10 volumes of ice-cold stopper was added, the cells were sedimented by centrifugation (14000*g* for 15 s at 24 °C), the supernatant was removed by aspiration, and the cells were resuspended in stopper and centrifuged again. The supernatant was removed by aspiration, and the cells were lysed in 500 μL of 3% perchloric acid. Precipitated proteins were sedimented, and aliquots of the clear supernatant were counted by liquid scintillation spectroscopy. Zero-time uptake was determined by addition of stopper to cells before addition of uptake medium. Cells were immediately processed as above. Non-protein-mediated uptake at 10 min was determined by addition of 50 μM CCB (a sugar transport inhibitor) to the uptake medium. Control experiments show that 3-*O*-methylglucose uptake at ice temperature occurs at a rate some 1000-fold lower than does uptake at 37 °C. Since 3-*O*-methylglucose transport by pigeon erythrocytes is symmetric (Simons, 1983a), this

means that sugar exit is reduced 1000-fold upon addition of ice-cold stopper and loss of intracellular radiolabel during a 4 min processing interval is less than 1%.

In some experiments, cells were poisoned by incubation in 8 μg/mL FCCP for 2 h at 37 °C. Control experiments included incubation in carrier (0.1−0.2% ethanol by volume) at 37 and 4 °C for 2 h. With poisoned cells, FCCP (or ethanol alone for control cells) was also included during the uptake assay. In experiments where transport was measured in the presence of cytochalasin B, control experiments also included the carrier (dimethyl sulfoxide) at the appropriate concentration (0.5% by volume).

Human red cell sugar transport measurements were as described by Helgerson et al. (1989). Sugar-free red cells or ghosts (at ice temperature) were exposed to 10 volumes of KCl medium (ice temperature) containing variable 3OMG concentrations and [^{14}C]3OMG (or variable D-glucose concentrations and [^{14}C]-D-glucose). Uptake was permitted to proceed for a fixed period of time (30 s) then 50 volumes (relative to the cell volume) of stopper solution was added to the cell suspension. The cells were sedimented by centrifugation (14000*g* for 3 min), washed once more in stopper, collected by centrifugation, and extracted in 500 μL of 3% perchloric acid. The acid extract was centrifuged, and duplicate samples of the clear supernatant were counted. Zero-time uptake points were obtained as described with avian red cells. Radioactivity associated with cells at zero time was subtracted from the activity associated with cells following the uptake period. All uptakes were normalized to equilibrium uptake where cells were exposed to sugar medium at 37 °C for 0.5−2 h prior to addition of stopper.

Estimates of rates of non-protein-mediated 3OMG uptake were made in parallel in each experiment in which cells were preincubated with 10 mM CCB [$K_{i(app)}$ for noncompetitive inhibition of uptake is 0.13 ± 0.01 μM]. CCB (10 μM) was also included in the uptake medium during these uptake measurements.

Net Sugar Exit Measurements. Avian erythrocytes were incubated in 100 μM [^{3}H]3OMG for 18 h at 4 °C. The half-time for 3OMG uptake by transbilayer diffusion under these conditions is 13 h. Sugar-loaded cells were placed on ice, and aliquots were sedimented by centrifugation (14000*g* for 1 min). The supernatant was removed and exit initiated by addition of 50 volumes (relative to the packed cell volume) of saline, and following the appropriate exit interval, exit was terminated by addition of 100 volumes of ice-cold stopper. Cells were sedimented by centrifugation, and the supernatant was aspirated and the cell pellet washed once more in 150 volumes of stopper. The final cell pellet was extracted in 500 μL of 3% perchloric acid and centrifuged, and duplicate samples of the clear supernatant were counted by liquid scintillation spectrometry. Zero-time exit points were obtained by addition of stopper to cells prior to addition of saline.

D-Glucose exit from human erythrocytes, from resealed erythrocyte ghosts, and from inside-out (human erythrocyte membrane) vesicles (IOVs) at 24 °C was measured by turbidimetry as described in Carruthers and Melchior (1983). The loss of intracellular sugar was monitored as altered apparent absorbance of a suspension of erythrocytes (0.2% cytocrit) following injection of sugar-loaded (60 mM) cells, ghosts, or IOVs into sugar-free saline. The time course of the change in apparent absorbance (measured at 420 mm) was digitized and recorded on magnetic media. The total absorbance change over the time course of exit is related linearly to the loss of 60 mM intracellular D-glucose (Carruthers & Melchior, 1983). The first derivative of the absorbance change (dA/dt; related directly to the rate of loss of D-glucose) was expressed as a function of the average absorbance over that dt [related to the average intracellular D-glucose concentration (Carruthers, 1990)]. V_{max} and $K_{m(app)}$ for exit were then obtained by direct, nonlinear regression analysis of these data, assuming that the rate of exit follows Michaelis−Menten kinetics.

Equilibrium Exchange 3-O-Methylglucose Uptake in Human Red Cells. In these experiments, intracellular [3OMG] = extracellular [3OMG] and radiolabeled 3OMG is used to monitor unidirectional sugar fluxes. Cells were first loaded with unlabeled 3-*O*-methylglucose at 37 °C as described above. Packed cells (10 mL) were aliquoted into tubes, and 100 mL of saline containing labeled sugar plus unlabeled sugar at a concentration identical to that of loaded cells was added to the cells. Totals were sampled, and transport was arrested at appropriate time intervals as described above. At 3OMG concentrations in excess of 0.1 mM, uptake of labeled sugar in equilibrium exchange experiments follows a simple exponential time course consisting of a leakage component characterized by rate constant k_l and a saturable or protein-mediated component characterized by rate constant k_{sat} (Cloherty et al., 1995). With S_t defined as the radioactivity associated with cells at time t and S_∞ as the radioactivity associated with cells at equilibrium, a plot of $\log[1-(S_t/S_\infty)]$ versus time is linear with slope $-(k_l + k_{sat})/2.3$. k_l can be estimated directly from log plots of uptake in the presence of 50 μM CCB (where k_{sat} over the 0.5−50 mM 3OMG range is reduced by more than 95%) versus time and the contribution of leakage to uptake subtracted from total uptake to calculate k_{sat}. k_l and k_{sat} were calculated from log plots of uptake by the method of least squares. Control experiments indicate that calculated $K_{m(app)}$ and V_{max} parameters for 3OMG equilibrium exchange entry are indistinguishable from those for equilibrium exchange exit.

Calculation of Michaelis and Velocity Parameters. [3OMG] versus uptake velocity data were linearized in the form [3OMG]/uptake versus [3OMG] to obtain estimates of V_{max} (1/slope) and $K_{m(app)}$ (x-intercept). These values were then used as initial estimates in a nonlinear regression analysis (KaleidaGraph 3.05; Synergy Software, Reading, PA) that assumes that transport is described by simple Michaelis−Menten kinetics and that solutions for $K_{m(app)}$ and V_{max} are acceptable when they change by less than 0.01% per iteration and the correlation coefficient for the fit is 0.9 or greater. Unless stated otherwise, $K_{m(app)}$ and V_{max} parameters are shown as mean ± SEM where the error refers to the standard error of the mean of parameters calculated in more than three separate experiments.

AMP-Activated Protein Kinase Assays. The AMP-activated kinase activity of extracts of erythrocytes and K562 cells were assayed as described (Davies et al., 1989). Cells were lysed in 10−20 volumes of ice-cold extraction medium, and the lysate was centrifuged at 14000*g* and 4 °C for 2 min. Aliquots of supernatant (2.5 μL) were added directly to 25 μL of kinase assay medium [containing SAMS peptide (200 μM), AMP (200 μM), and [γ-^{32}P]ATP (200 μM, 500 cpm/pmol)]. After 10 min of incubation at 30 °C, 15 μL aliquots were removed and spotted on squares of P81,

Whatman phosphocellulose paper which were then dropped into 500 mL of 1% H_3PO_4. The squares were washed for 20 min in three changes of H_3PO_4 and then were washed once in acetone. The squares were air-dried and counted by liquid scintillation spectrometry.

Analytical Procedures. Protein was determined by the Pierce BCA procedure. SDS−PAGE (10%) of membrane proteins and GLUT1 were as described previously (Carruthers & Helgerson, 1989). ATP was assayed by the method of Adams (1963). This procedure is not specific for ATP. GTP, UTP, and ITP are also read as ATP but in combination are present at less than 6% of erythrocyte ATP content (Bartlett, 1959).

RESULTS

Effects of Stimulation on Net Transport. Sugar uptake by avian red cells is stimulated following cellular exposure to metabolic poisons (Diamond & Carruthers, 1993). Because protein-mediated sugar transport is virtually absent in freshly isolated pigeon red cells, our previous studies (Diamond & Carruthers, 1993) could not determine whether V_{max}, $K_{m(app)}$, or both V_{max} and $K_{m(app)}$ for 3-*O*-methylglucose uptake by avian erythrocytes are affected during transport stimulation by FCCP. As the half-time for development of full transport stimulation by FCCP (37 °C, 4 μg of FCCP/mL^{-1}) is approximately 40 min, it is possible to sample cells at various times following exposure to FCCP and to accurately determine the concentration dependence of sugar uptake. Figure 1A summarizes the results of our measurements. We conclude that $K_{m(app)}$ for protein-mediated 3OMG uptake (when measurable) is not affected significantly by metabolic poisoning whereas V_{max} for 3OMG uptake extends over a 40-fold range.

GLUT1-mediated net D-glucose and 3OMG transport in human erythrocytes are accelerated upon dilution of cytosolic ATP content by lysis/resealing in KCl medium (Carruthers, 1986a; Carruthers & Melchior, 1983). This effect is reversed by including ATP in the resealing medium (Helgerson et al., 1989). Figure 1B shows that, as cytosolic ATP content is progressively diluted, $K_{m(app)}$ and V_{max} for net D-glucose efflux from human erythrocyte inside-out-membrane vesicles and V_{max} for net exit from human erythrocyte ghosts increase while $K_{m(app)}$ for net exit from erythrocyte ghosts falls.

Effects of Stimulation on Exchange Transport. Pigeon erythrocytes were loaded with varying concentrations of 3OMG by overnight incubation at 4 °C. Under these conditions, the larger portion of sugar uptake occurs via slow transbilayer sugar diffusion with a $t_{1/2}$ of 13 h (Diamond & Carruthers, 1993). In some experiments, radiolabeled 3OMG was also included in the incubation medium to permit sugar efflux determinations or to facilitate determination of intracellular [3OMG] when incubation was arrested the following morning.

3OMG uptake is accelerated by overnight cell loading with 3OMG (Figure 2A). Half-maximal stimulation of uptake is observed at 3.7 ± 0.6 mM intracellular 3OMG (n = 3). Cytochalasin B inhibitable 3OMG exit is barely measurable in normoxic cells. Addition of extracellular unlabeled 3OMG stimulates exit markedly (Figure 2B). Subsequent exposure of sugar-loaded cells to FCCP does not further stimulate uptake, and exit into 3OMG-containing medium is not further increased upon cellular metabolic depletion.

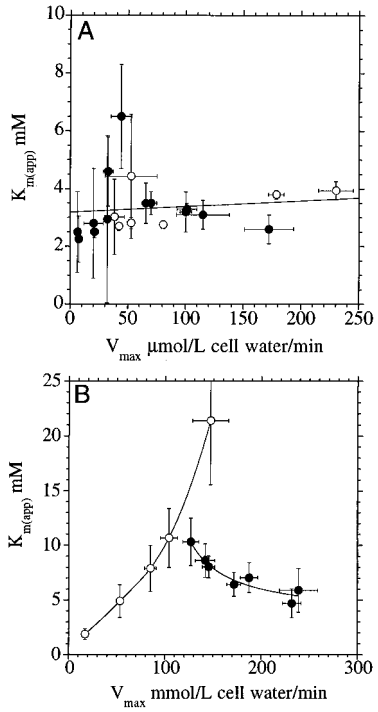

FIGURE 1: Effect of transport stimulation on $K_{m(app)}$ and V_{max} for uptake of 3-*O*-methylglucose by avian red cells (A) and for uptake and exit of D-glucose by human erythrocytes (B). (A) The ordinate is $K_{m(app)}$ for 3OMG uptake in millimolar. The abscissa is V_{max} for 3OMG uptake at 37 °C in micromoles per liter of cell water per minute. Filled symbols are data from this laboratory. Results are shown as mean ± SEM of the computed $K_{m(app)}$ and V_{max} for sugar uptake in each experiment using six or more 3OMG concentrations over the 0.5−50 mM range. Open symbols represent data from Simons (1983a). The line drawn through the points was computed by the method of least squares. (B) Variation in $K_{m(app)}$ and V_{max} for D-glucose exit from human erythrocyte ghosts (●) and from human erythrocyte inside-out membrane vesicles (○) in which the cytosolic surface of the cell membranes is exposed to varying concentrations of ATP. The ordinate is $K_{m(app)}$ for D-glucose exit in millimolar. The abscissa is V_{max} for D-glucose exit in millimoles per liter of cell water per minute. Sealed IOVs were exposed (○, from left to right on the plot) to 2, 0.5, 0.25, 0.05, or 0 mM exogenous Mg·ATP. Ghosts (●, from left to right on the plot) were resealed in the presence of 2, 0.4, 0.2, 0.15, 0.1, 0.05, and 0 mM exogenous Mg·ATP. Results are shown as mean ± SEM of the results of four or more determinations. The curves drawn through the points have no theoretical significance.

These observations suggest that, unlike the net (uniport) sugar transport capacity of the cell, the exchange transport capacity of the pigeon erythrocyte sugar transporter is insensitive to FCCP.

Equilibrium exchange 3OMG transport by human erythrocyte ghosts at 24 °C was measured in the presence and absence of 4 mM intracellular ATP. Equilibrium exchange uptake was also measured in intact cells. V_{max} for exchange transport in intact cells and ATP-containing and ATP-free ghosts are 248 ± 32, 214 ± 19, and 236 ± 29 mmol·(L of cell water)$^{-1}$·min^{-1}, respectively (n = 3). $K_{m(app)}$ for exchange transport in intact cells and ATP-containing and ATP-free ghosts are 28.6 ± 2.4, 34.1 ± 6.3, and 58.6 ± 7.5 mM, respectively (n = 3).

Is the Availability of the Export Site Regulated? Sugar uptake by normoxic avian red cells could be inhibited because oxidative phosphorylation leads to the production of nontransportable, intramolecular species that bind reversibly to the sugar export site of the glucose transporter. This

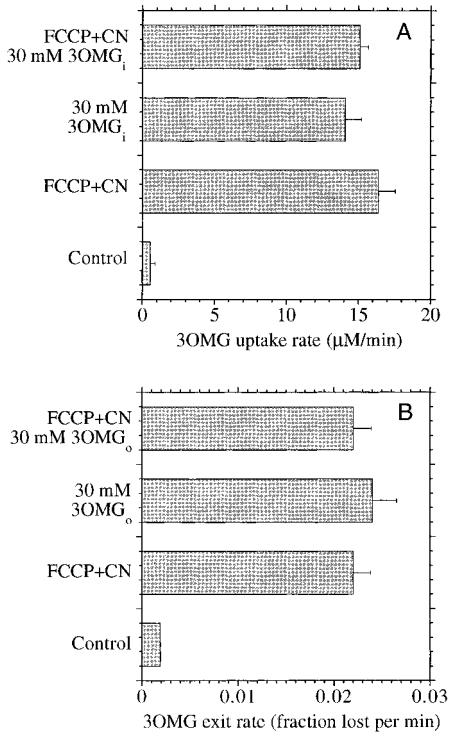

FIGURE 2: Transacceleration in avian red cells. Avian red cells were exposed overnight at 4 °C to saline, to saline containing unlabeled 3-*O*-methylglucose, or to saline containing [^{14}C]-3-*O*-methylglucose. The half-time for sugar loading by diffusion at this temperature is 13 h. Cells were then washed in saline and stored on ice. (A) Uptake of 1 mM [^{14}C]-3-*O*-methylglucose was measured in control cells, in cells that were exposed overnight to unlabeled 40 mM 3OMG, and in both groups of cells following metabolic depletion using 2 mM NaCN plus 8 μg/mL FCCP for 2 h at 37 °C. Fractional equilibration of sugar-loaded cells with extracellular sugar was 76%. (B) Cells incubated with labeled 3OMG were washed, and sugar efflux into saline or into saline plus 30 mM unlabeled 3OMG was monitored before and after 2 h of metabolic depletion using 2 mM NaCN plus 8 μg/mL FCCP. The number of measurements per condition is four or more. Results are shown as mean ± SEM.

type of transport regulation by deinhibition has been suggested for anisomycin stimulation of sugar transport in 3T3-L1 adipocytes (Clancy et al., 1991; Czech et al., 1992). Such a molecule, like the transport inhibitor cytochalasin B, would noncompetitively inhibit sugar uptake but competitively inhibit sugar exit (Basketter & Widdas, 1978). This molecule would competitively inhibit cytochalasin B and forskolin binding to GLUT1 because cytochalasin B and forskolin also bind at or close to the sugar export site (Basketter & Widdas, 1978; Sergeant & Kim, 1985). One prediction of this hypothesis is that, as transport becomes desuppressed (due to the loss of endogenous inhibitor), $K_{i(app)}$ for cytochalasin B and forskolin noncompetitive inhibitions of sugar uptake will fall.

We therefore prepared pigeon erythrocytes with varying extents of transport desuppression (0, 10, 30, and 60 min exposure to FCCP) and determined $K_{i(app)}$ for 3OMG uptake inhibition by cytochalasin B and by forskolin. Our results are summarized in Figure 3A and demonstrate that the apparent affinity of the avian erythrocyte sugar exit site for cytochalasin B and forskolin is unchanged by transport desuppression. We conclude that transport suppression does not result from the presence of an intracellular species that competitively occupies the sugar exit site.

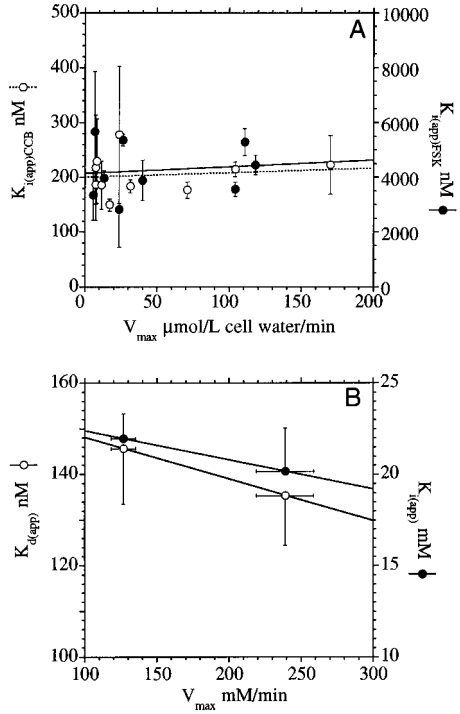

FIGURE 3: (A) Effects of transport stimulation on $K_{i(app)}$ for cytochalasin B (○) and forskolin (●) inhibition of 3OMG uptake by avian red cells. The ordinate is $K_{i(app)}$ for inhibition of 1 mM 3OMG uptake. The abscissa is V_{max} for 3OMG uptake (micromoles per liter of cell water per minute) obtained in parallel experiments. Results are shown as mean ± SEM for the computed $K_{i(app)}$ in each experiment. Inhibition titration curves were obtained by using at least eight inhibitor concentrations in each experiment. **(**B) Effects of intracellular ATP on CCB and D-glucose binding to human erythrocyte ghosts. The ordinate is $K_{d(app)}$ for CCB binding (○) to human erythrocyte ghosts in nanomolar and $K_{i(app)}$ for D-glucose inhibition (●) of CCB binding to human red cell ghosts in millimolar. The abscissa is V_{max} for D-glucose exit in millimoles of D-glucose per liter of cell water per minute. Ghosts were prepared in the presence (left-most data points; V_{max} exit = 130 mM D-glucose per minute) and absence of 2 mM Mg·ATP (right-most data points; V_{max} exit = 240 mM D-glucose per minute). Results are shown as mean ± SEM for computed $K_{d(app)}$ and $K_{i(app)}$ in each experiment. Straight lines drawn through the data points of A and B were computed by the method of least squares.

Similar results are obtained with human erythrocyte ghosts resealed in the presence of varying ATP concentrations. Although net sugar uptake and exit in ATP-depleted erythrocyte ghosts are stimulated, neither $K_{d(app)}$ nor B_{max} for cytochalasin B binding to red cell GLUT1 is affected (Figure 3B). Moreover, $K_{i(app)}$ for D-glucose inhibition of cytochalasin B binding to human erythrocytes is unchanged by ATP depletion (Figure 3B).

Studies with Erythrocyte Ghosts. Pigeon erythrocytes were hypotonically lysed in lysis medium and resealed by incubation for 40 min at 37 °C in pigeon saline containing ATP (8 mM), AMP-PNP (4 mM), GSH (2 mM), or no exogenous materials. Cells were then collected, and cytochalasin B inhibitable 3OMG uptake was measured with or without prior cellular exposure to FCCP. Sugar import stimulation by FCCP is reconstituted when cells are resealed in the presence of ATP and/or GSH but not when cells are loaded with AMP-PNP (Table 1). Basal sugar transport is unaffected by these agents. The action of ATP is lost when hypotonically lysed cells are resealed in the additional presence of Ca^{2+} (Table 1). This effect is most likely indirect, resulting from Ca^{2+} activation of ATPases which deplete the ATP content of the

Table 1: Reconstitution of Sugar Transport Regulation[a]

additions	cytochalasin B inhibitable 3OMG uptake[b]			
	pigeon red cells		human red cells	
	control[c]	FCCP[d]	control[c]	FCCP[d]
intact cells				
none	0.3 ± 0.0	2.9 ± 0.1[e]	21.5 ± 1.9	20.6 ± 2.2
extracellular ATP (8 mM)	0.2 ± 0.0	3.2 ± 0.2[e]	19.1 ± 1.8	
extracellular GSH (2 mM)	0.2 ± 0.0	1.0 ± 0.1[e,f]	2.4 ± 1.6[f]	
resealed ghosts				
none	1.1 ± 0.1	1.0 ± 0.1	7.6 ± 0.5[g]	
intracellular ATP (8 mM)[h]	0.8 ± 0.1[f]	1.6 ± 0.1[e]	23.4 ± 2.1	
intracellular GSH (2 mM)	0.6 ± 0.1[f]	3.5 ± 0.1[e]	7.2 ± 1.3[g]	
intracellular ATP + GSH[i]	0.4 ± 0.1[f]	2.0 ± 0.2[e]		
intracellular AMP−PNP (4 mM)	0.9 ± 0.1	1.0 ± 0.1	23.7 ± 1.9	
intracellular ATP + Ca[j]	1.0 ± 0.1	0.9 ± 0.1	14.2 ± 0.9[g]	
intracellular GSH + Ca[j]	1.2 ± 0.1	0.9 ± 0.1		
extracellular ATP (8 mM)	0.8 ± 0.2	0.9 ± 0.1		
extracellular GSH (2 mM)	0.9 ± 0.1	1.0 ± 0.1	4.8 ± 0.3[f]	

[a] Effects of various agents on sugar transport in pigeon and human erythrocytes. [b] Protein-mediated (50 μM CCB inhibitable) uptake of 0.1 mM 3OMG (pigeon red cells, 37 °C; human red cells, 4 °C) in micromoles per liter of cell water per minute. Results are shown as mean ± SEM of three or more determinations. Uptake was measured following 2 h of incubation at 37 °C. [c] Without 8 μg/mL FCCP. [d] With 8 μg/mL FCCP. [e] This result is significantly greater ($p < 0.005$; one-tailed t test) than the corresponding control condition. [f] This result is significantly less ($p < 0.005$; one-tailed t test) than that obtained upon no addition of agents. [g] This results is significantly less than that obtained using untreated intact human red cells. [h] The ATP concentration when resealing human red cells was 4 mM. [i] This addition contained 8 mM ATP plus 2 mM GSH. [j] The Ca^{2+} concentration was 100 μM.

medium from 8 to 0.3 mM during the course of resealing. Extracellular GSH inhibits FCCP stimulation of sugar transport in intact cells (Table 1), suggesting that the site of GSH action to restore FCCP stimulation of transport in ghosts may be intracellular.

ATP and AMP-PNP restore normal 3OMG transport to ATP-free human erythrocyte ghosts (Table 1). Reduced glutathione is without effect on 3OMG uptake when present only inside human erythrocyte ghosts (Table 1).

AMP-activated kinase is activated in hepatocytes following cellular exposure to hypoxia or to metabolic inhibitors (Davies et al., 1989). We therefore measured the AMP-activated kinase activity of cytosol obtained from pigeon erythrocytes using the SAMS synthetic peptide (Davies et al., 1989) as substrate for the assay. The time course of sugar transport stimulation by CN/FCCP closely matches the time course of ATP depletion and activation of AMP-dependent kinase in red cell cytosol (Figure 4). Owing to the very low level of GLUT1 expression in avian erythrocytes, it is not possible to use immunoprecipitation techniques to demonstrate stoichiometric ligand incorporation into red cell-resident GLUT1 (Diamond & Carruthers, 1993). However, we have evaluated the ability of cytosol from FCCP-poisoned avian erythrocytes to phosphorylate exogenous, purified GLUT1 by substitution of purified GLUT1 for peptide in the AMP-activated kinase assay. GLUT1 is not phosphorylated by avian erythrocyte cytosol that contains activated AMP-dependent kinase activity ($n = 2$).

AMP-dependent kinase is not activated and cellular ATP levels are not reduced by overnight loading of pigeon erythrocytes with 3-*O*-methylglucose (Table 2). Human erythrocytes lack detectable AMP-activated kinase activity (Table 2). Exposure of human K562 cells to FCCP leads to activation of AMP-activated kinase activity, but net sugar uptake is unchanged (Table 2).

DISCUSSION

Several studies demonstrate that net sugar transport is virtually absent in normoxic pigeon red cells but is stimulated markedly upon cellular exposure to anoxia or mitochondrial inhibitors (Carruthers & Simons, 1978; Cheung et al., 1977; Simons, 1983a,b; Whitfield & Morgan, 1983; Wood & Morgan, 1969). We have demonstrated that the absence of transport in normoxic cells does not result from intracellular sequestration of glucose transporters but rather from catalytic suppression of cell surface sugar transporter (GLUT1) function (Diamond & Carruthers, 1993). We concluded that transport stimulation results from desuppression of cell-surface GLUT1. The results of the present study support the hypothesis that the avian erythrocyte sugar transporter retains antiporter function under basal conditions and attains uniport capacity as the cell becomes metabolically depleted. As an antiporter, the carrier cannot catalyze net sugar import or exit. As a uniporter, the carrier can now catalyze net sugar transport which allows the cell to exploit extracellular glucose as a source of metabolic fuel. Transport stimulation in conjunction with the release of glycolysis from aerobic restraint can result in increased synthesis of glycolytic ATP. The signal for antiporter/uniporter switching could be AMP-activated protein kinase which is uniquely sensitive to the energy status of cytosol (Moore et al., 1991). If this hypothesis is correct, the molecular species that control antiporter/uniporter switching are downstream from AMP-activated kinase and are expressed functionally only in cells characterized by this form of sugar transport regulation.

Avian erythrocyte GLUT1 may be the first characterized example of an antiporter/uniporter switch mechanism. The pigeon red cell transporter may also be an extreme instance of this switch because, although protein-mediated sugar transport is not measurable in freshly isolated pigeon erythrocytes, saturable sugar transport is reported in normoxic erythrocytes from geese and ducks (Cheung et al., 1977; Wood & Morgan, 1969). This low-level, saturable sugar sugar transport is subsequently stimulated upon cell exposure to cyanide (Cheung et al., 1977; Wood & Morgan, 1969). Varying degrees of "slippage" have been reported for other antiporters that allow low levels of uniport function (Aronson, 1985; Fröhlich, 1984; Kaplan et al., 1983). Indeed,

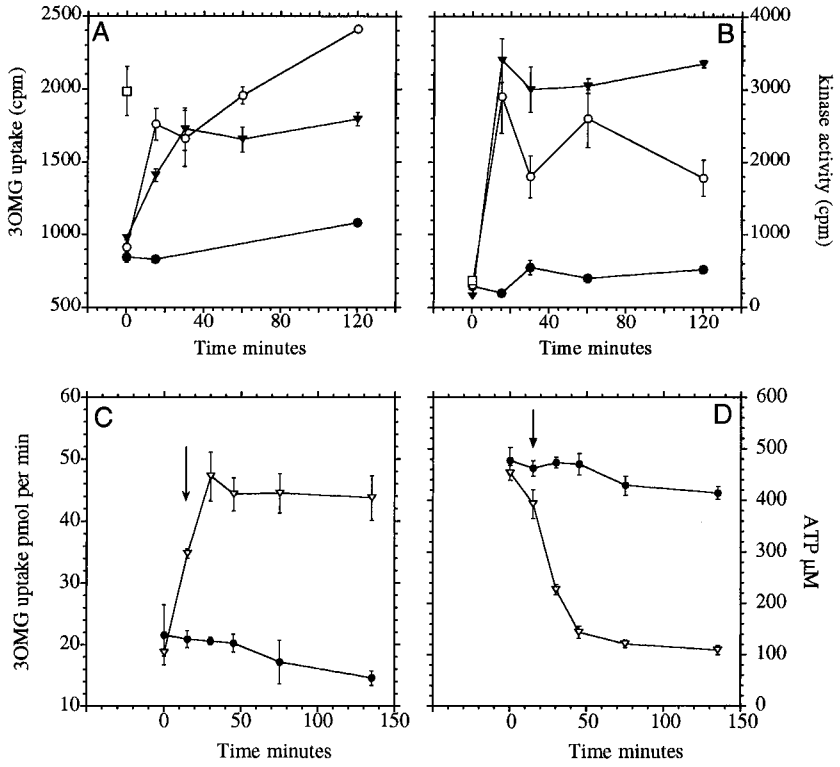

FIGURE 4: Time course of 3OMG transport stimulation (A and C), AMP-activated kinase activation (B), and cellular [ATP] reduction (D) in pigeon red cells produced by cyanide (○; 2 mM), by FCCP (▼; 8 μg·mL^{-1}), or by cyanide plus FCCP (▽). Data in A and B represent paired experiments. Data in C and D are paired experiments. Cells were exposed to saline (●) or to saline containing poison (○, ▼, ▽) either at zero time (A and B) or at the time indicated by the arrow (C and D), and aliquots were taken at various times for assay of [^{14}C]3OMG uptake (A and C in counts per minute per 10 min), AMP-activated kinase activity (B, counts per minute of ^{32}P incorporation into peptide substrate), or cellular extract [ATP] (D in micromolar). Results are shown as mean ± SEM of three determinations. Results are also shown for 3OMG uptake (A) and AMP-activated kinase activity (B) of cells that were incubated overnight in 40 mM unlabeled 3OMG (□).

Table 2: Sugar Transport and Cellular Metabolic Status

	3OMG uptake[a]	[ATP] (mM)	AMP-activated kinase[b]
pigeon red cells			
control	0.07 ± 0.01	1.91 ± 0.11	624 ± 35
FCCP[c]	0.81 ± 0.05[f]	0.41 ± 0.04[g]	3392 ± 161[f]
overnight at 4 °C[d]	0.10 ± 0.02	1.28 ± 0.08	518 ± 84
overnight at 4 °C plus 3OMG[d]	1.20 ± 0.10[f]	1.21 ± 0.08	473 ± 22
human red cells			
control	19.4 ± 2.6	2.61 ± 0.32	2177 ± 280
overnight at 37 °C + D-glucose[e]	18.7 ± 1.4	2.42 ± 0.18	2063 ± 196
overnight at 37 °C − D-glucose[e]	8.3 ± 1.4[g]	0.85 ± 0.07[g]	2144 ± 120
K562 cells			
control	16.8 ± 0.1	1.59 ± 0.16	2663 ± 282
FCCP[c]	5.6 ± 0.2[g]	0.12 ± 0.05[g]	6224 ± 542[f]

[a] 3OMG uptake in micromoles of 3OMG per liter of cell water per minute was measured in pigeon red cells at 37 °C and in human red cells and K562 cells at 4 °C. [b] AMP-activated kinase activity is expressed as SAMS peptide-dependent, ^{32}P incorporation per 2.5 μL of cell extract per 10 min. [c] FCCP concentration was 8 μg/mL, and exposure was for 2 h. [d] Cells were incubated at 4 °C overnight (18 h) with or without 40 mM 3OMG. [e] Cells were incubated at 37 °C overnight (18 h) with or without 5 mM D-glucose. The cells were then incubated at 20 °C for 10 min in 50 volumes of glucose-free medium. [f] This result is significantly greater than the control value ($p < 0.005$; one-tailed t test). [g] This result is significantly lower than the control value ($p < 0.005$; one-tailed t test). Results are shown as mean ± SEM of three or more determinations.

human erythrocyte sugar transport at 4 °C is characterized by net sugar uptake that proceeds some 50-fold more slowly than does exchange sugar uptake (Cloherty et al., 1996).

The switch from antiporter to uniporter could be explained by two hypothetical mechanisms for sugar transport. According to the "simple carrier" mechanism, the transporter alternately presents sugar import (e2) and sugar export (e1) sites but never both simultaneously (Stein, 1986; Widdas, 1952). In the antiporter mode, the simple carrier cannot undergo conformational change between import (e2) and export (e1) competent states unless sugar is bound to the carrier. Substrate-dependent conformational change is called translocation (Stein, 1986). In the uniport mode, the simple carrier can undergo e1 ↔ e2 conformational change in the absence of bound sugar. Substrate-independent conformational change is called relaxation or slippage (Stein, 1986) and may occur more rapidly, more slowly, or at a rate indistinguishable from translocation (Widdas, 1952). In the absence of any change in translocation, the simple carrier model predicts that both $K_{m(app)}$ and V_{max} for sugar import increase in proportion with the first order rate constant (k_o) for e1 → e2 relaxation (Stein, 1986). As relaxation is

accelerated, the carrier is converted from an antiporter to a uniporter, $K_{m(app)}$ and V_{max} for sugar import are increased, and net sugar import is stimulated. Our measurements demonstrate, however, that $K_{m(app)}$ for import is unchanged while V_{max} for import is increased by as much as 40-fold.

What could account for this divergence between theory and observation? If translocation and relaxation were each stimulated by cellular metabolic depletion to the same extent, this would result in increased V_{max} for import but no change in $K_{m(app)}$. This, however, is inconsistent with the observation that maximum rates of sugar import (sugar uniport) in metabolically poisoned cells are very similar to rates of exchange sugar uptake (sugar antiport) in both basal and poisoned cells. In other words, translocation rates appear unchanged but relaxation rates are increased. We therefore conclude that the theoretical, simple carrier mechanism for sugar transport cannot account for these observations.

A more complex model for sugar transport called the "two-site" carrier (Carruthers, 1991) is more successful in explaining our observations. This mechanism describes transport via a carrier that can bind extracellular and intracellular sugars simultaneously. In order to explain the findings of the present study, the hypothetical two-site carrier of basal cells cannot undergo translocation unless sugars are bound at both import and export sites simultaneously. With this constraint, the carrier functions as an antiporter. In the metabolically depleted cells, this constraint is lifted and translocation can proceed even when only one site is occupied. Since the maximum rate of two-site carrier-mediated sugar transport is dependent upon translocation rates alone (Carruthers, 1991), there is no *a priori* reason why V_{max} cannot change in the absence of an altered $K_{m(app)}$ for transport.

A more trivial explanation for our results is that overnight exposure of avian red cells to 3-*O*-methylglucose results in cellular metabolic depletion. This could result from contamination of 3-*O*-methylglucose by a low-abundance molecular species that interferes in some way with cellular metabolism. This hypothesis is inconsistent with the unchanged AMP-activated kinase activity [a sensitive indicator of the energy charge of cytosol (Moore et al., 1991)] of 3-*O*-methylglucose-loaded pigeon red cells. It is also possible that 3-*O*-methylglucose interacts in some way with hypothetical, intracellular regulatory species that normally suppress the transport mechanism. This possibility is not eliminated by the present studies and is consistent with our observation (Cloherty et al., 1995) that human red cells contain an intracellular sugar (3-*O*-methyglucose) binding complex in functional proximity to the glucose transporter.

The molecular factors that the signal cellular metabolic status to the glucose transporter are not known. It has been suggested that sugar transport in tissues where transport is rate-limiting for glucose utilization is inversely proportional to cellular ATP levels (Elbrink & Bihler, 1975; Randle & Smith, 1958). However, in most tissues, it is possible to demonstrate significantly elevated rates of sugar transport under circumstances where total cellular ATP levels are not significantly changed (Holloszy & Narahara, 1975; Morgan et al., 1961). We now know that AMP-dependent protein kinase can become activated by cellular exposure to poisons under circumstances where total ATP levels are unchanged (Moore et al., 1991). Indeed, we observe that activation of sugar transport and AMP-dependent kinase in pigeon erythrocytes are closely correlated. However, AMP-dependent kinase is also stimulated in GLUT1-expressing cells under circumstances where GLUT1-mediated sugar transport is unchanged. These cells are characterized by very high basal rates of sugar uniport. We conclude therefore that avian erythrocytes express a factor(s) that suppresses GLUT1-mediated uniport and that this factor is absent in cells with high levels of basal transport. We hypothesize that this factor noncompetitively (allosterically) inhibits GLUT1 uniport but upon cellular metabolic depletion is covalently modified directly or indirectly by AMP-dependent protein kinase, causing its dissociation from GLUT1 and subsequent release of the transporter from uniport suppression.

Regulation of Human Erythrocyte Sugar Transport. Unlike sugar transport in pigeon red cells, sugar transport (uniport) in human erythrocytes is present under basal conditions. This may reflect the fact that human erythrocytes are incapable of aerobic metabolism and derive most of their metabolic fuel from glycolysis (Gutierrez et al., 1992). However, the human erythrocyte glucose transport system is subject to a different form of acute regulation. GLUT1 is a nucleotide binding protein whose conformational state, substrate binding properties, and catalytic turnover are markedly affected upon of binding intracellular ATP (Carruthers, 1986a,b; Carruthers et al., 1989; Carruthers & Helgerson, 1989; Carruthers & Melchior, 1983; Hebert & Carruthers, 1986). ATP binding to GLUT1 reduces $K_{m(app)}$ and V_{max} for sugar import, increases $K_{m(app)}$ and reduces V_{max} for sugar exit, and reduces $K_{m(app)}$ for exchange transport. This action of ATP is allosteric since it does not require nucleotide hydrolysis and is reversed competitively upon addition of AMP or ADP (Carruthers & Helgerson, 1989).

We made two unexpected observations in the present study. The first is that cytochalasin B binding to the sugar transport exit site is unaffected by omission of cytosolic ATP. The second is that $K_{i(app)}$ for sugar inhibition of cytochalasin B binding at this site is unaffected by ATP. We expected that, as with $K_{m(app)}$ for sugar exit, $K_{d(app)}$ for cytochalasin B binding and $K_{i(app)}$ for D-glucose inhibition of cytochalasin B binding would be increased by ATP. This is even more surprising when similar experiments that measure the effect of ATP on sugar binding to membrane-resident GLUT1 show that ATP increases $K_{d(app)}$ for sugar binding to a low-affinity (presumed) exit site in the transporter and reduces $K_{d(app)}$ for sugar binding to a high-affinity (presumed) import site in the transporter (Carruthers, 1986a,b).

We have previously argued against the hypothesis that human erythrocyte sugar transport is mediated by an asymmetric simple carrier mechanism (Carruthers & Helgerson, 1991; Helgerson & Carruthers, 1987), while our findings support (but do not prove) the hypothesis that transport is mediated by a two-site carrier mechanism. We now know that the apparent steady-state kinetics of human erythrocyte sugar transport reflect two serial processes: transmembrane sugar transport and sugar binding to and dissociation from an intracellular sugar binding complex (Cloherty et al., 1995). In addition, the transport process is intrinsically more symmetric than previously believed (Cloherty et al., 1996). It is, therefore, neither possible nor useful to model a transport process in detail when the detailed behavior of the translocation process is not directly measurable. However, the experimental findings are broadly modeled by a two-site carrier mechanism when (1) ATP reduces $K_{d(app)}$ for sugar

binding to the sugar import site, (2) ATP increases $K_{d(app)}$ for sugar binding to the sugar export site only when the import site is occupied with sugar, (3) ATP inhibits translocation of externally bound sugar only when the trans export site is not occupied by sugar, (4) the intrinsic tryptophan fluorescence of the transporter is quenched more significantly when the transporter is liganded by both extra- and intracellular sugar, and (5) sugar dissociation from the intracellular sugar binding complex is slowed by the absence of ATP.

It is unclear at this time whether the low sugar uniport function (relative to antiporter function) of human erythrocytes reflects directly the rate of catalytic turnover of the glucose carrier or the indirect effects of intracellular sugar complexation on our ability to measure transport accurately (Cloherty et al., 1996). If human GLUT1 uniport activity in human red cells is truly lower than antiporter activity and ATP depletion does stimulate uniport directly, this would suggest that sugar transport regulation in human and avian erythrocytes is expressed through a common uniport/antiport switch principle but that the molecular details of the signaling pathways differ.

Conclusions. Under basal conditions, human and avian erythrocyte unidirectional sugar uniport proceed significantly more slowly than unidirectional sugar antiport. Following metabolic depletion, rates of sugar uniport approach rates of antiport. In avian red cells, stimulation of antiport requires intracellular ATP while uniport acceleration in human red cells requires the loss of intracellular ATP.

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