ATP Regulation of the Human Red Cell Sugar Transporter*

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Anthony Carruthers

From the Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Purified human red blood cell sugar transport protein, an intrinsic tryptophan fluorescence is quenched by D-glucose and 4,6-ethylidene glucose (sugars that bind to the transport), phloretin and cytochalasin B (transport inhibitors), and ATP. Cytochalasin B-induced quenching is a simple saturable phenomenon with a dissociation constant of 0.15 μM and a maximum capacity of 0.85 cytochalasin B binding sites per transporter. Sugar-induced quenching consists of two saturable components characterized by low and high dissociation constants. These binding sites appear to correspond to influx and efflux transport sites, respectively, and coexist within the transporter molecule. ATP-induced quenching is also a simple saturable process with a dissociation constant of 50 μM. Indirect estimates suggest that the ratio of ATP-binding sites per transporter is 0.87:1. ATP reduces the low dissociation constant and increases the high dissociation constant for sugar-induced fluorescence quenching. This effect is half-maximal at 45 μM ATP. ATP produces a 4-fold reduction in the dissociation constant and a 2.4-fold reduction in the dissociation constant for cytochalasin B-inhibitable D-glucose efflux from inside-out red cell membrane vesicles (IOVs). This effect on transport is half-maximal at 45 μM ATP. AMP, ADP, α,β-methyleneadenosine 5′-triphosphate, and β,γ-methyleneadenosine 5′-triphosphate at 1 mM are without effect on efflux of D-glucose from IOVs. ATP modulation of the dissociation constant for D-glucose efflux from IOVs is immediate in onset and recovery. ATP inhibition of the dissociation constant for D-glucose exit is complete within 5-15 min and is only partly reversed following 30-min incubation in ATP-free medium. These findings suggest that the human red cell sugar transport protein contains a nucleotide-binding site(s) through which ATP modifies the catalytic properties of the transporter.

Insulin stimulation of sugar transport in muscle and adipose seems not to result from increased intrinsic activity of plasmalemmal sugar transport proteins but rather from an insulin-induced "recruitment" of sugar transport protein from microsomal membranes to the plasma membrane (1-4). Anoxia, metabolic depletion, and contractile activity also markedly stimulate sugar transport in skeletal muscle (5, 6). However, a number of lines of evidence suggest that insulin-independent acceleration of skeletal muscle sugar transport is mediated by recruitment-independent mechanisms.

Insulin-induced, recruitment-mediated sugar transport stimulation is abolished in metabolically depleted adipocytes (4). It seems unlikely, therefore, that acceleration of skeletal muscle sugar transport by metabolic depletion could result from an energy-dependent recruitment-like mechanism. Moreover, whereas insulin stimulation of sugar transport is both rapid in onset and recovery (4), the rapid stimulation of sarcoplasmic sugar transport by metabolic depletion and contractile activity recovers only very slowly upon restoration of resting sarcoplasmic ATP and Ca²⁺ levels (5, 6). Is sugar transport also subjected to a recruitment-independent form of regulation in which the direct covalent or allosteric modification of plasmalemmal sugar transport protein results in altered catalytic activity? Direct support for this hypothesis is unavailable. Indirect support has been obtained from studies of adipose and human erythrocyte sugar transport. In adipose, a variety of agents that act to increase cytosolic cAMP levels also inhibit sugar transport without altering the distribution of carrier between plasmalemmal and microsomal compartments (7). Human erythrocyte sugar transport is modulated by low molecular weight factors present in cytosol (8), possibly ATP (9). The erythrocyte — lacking the specialized intracellular organelles necessary for membrane cycling, is, of course, incapable of carrier recruitment. The red cell therefore provides almost the unique potential for the study of recruitment-independent sugar transport regulation under conditions where physiological effects on transport are not obscured by the possible occurrence of carrier redistribution between plasmalemmal and intracellular compartments.

This study demonstrates that micromolar concentrations of ATP drastically modify sugar transport in human red cell inside-out vesicles and substrate binding to purified human red cell sugar transport protein.

EXPERIMENTAL PROCEDURES

Materials—Outdated, whole human blood was obtained from the University of Massachusetts Medical Center Blood Bank.

Solutions—Tris medium consisted of 50 mM Tris-HCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.0 using 1 M Tris base.

Preparation of Inside-out Vesicles (IOVs)—IOVs were formed and loaded with 60 mM D-glucose as described previously (8). Sialic acid accessibility assays were performed as described by Steck and Kant (10) and sialic acid released quantitated by the method of Warren (11). 95% of membrane sialic acid groups in IOVs are inaccessible to the enzyme sialidase.

Preparation of Purified Band 4.5 Protein—Band 4.5 was purified from red cell ghosts as described previously (12-15). The final preparation, which consists of unsealed protein/lipid/residual Triton X-100 particles (molar ratio = 10:100,000:5), permeability to D-glucose and H₂O = 1,000-5,000-fold greater than permeabilities in IOVs (16) was aliquoted as samples containing 50 µg of band protein and stored at -70 °C.

Fluorescence Measurements—Fluorescence measurements were performed at 23 °C using a Farrand Spectrofluorimeter MK 2 with excitation at 285 nm and emission bandwidths of 2.5 and 10 nm, respectively. Band 4.5 samples (25 µl in 100 µl) were

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1The abbreviations used are: IOV, inside-out vesicle; AMP-CPP, α,β-methyleneadenosine 5′-triphosphate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; NATA, N-acetyltryptophanamide; CGIC, ethylenediamine; CCB, cytochalasin B.
added to 2 ml of Tris medium (final transporter concentration, 0.22 mM). Sugars, nucleotides, and transport inhibitors (stock solutions adjusted to pH 7.0 using 1 M Tris base) were injected into the cuvette from above. The contents of the cuvette were constantly stirred using a Spectrocoll Inc. cuvette stirrer. Fluorescence quenching data represent quenching of steady-state emission at 234 nm. In all instances, the cuvettes for steady-state emission and fluorescence decayed monoexponentially (τ = 4–8 min at 23 °C) due, presumably, to photoysis of tryptophan residues.

Steady-state quenching data are subject to the following systematic errors: 1) Dilution effects resulting from the addition of volumes of stock ligand solution; 2) changes in emission spectral position/shape upon interaction of transporter with ligand; 3) changes in transporter absorption spectrum upon interaction with ligand; 4) variations in the refractive index of the transporter solution upon addition of ligand; 5) the inner filter effect (attenuation of the exciting light due to absorption at 295 nm by added ligand); 6) reabsorption of transporter fluorescence by ligand. Dilution corrections were assessed using the ligands L-glucose, mannitol, and cytochalasin B and D. These agents do not interact significantly with the transport protein, and following correction for dilution effects at maximum ligand concentrations, emission was within 98–100% of control emissions. Sugars that interact with the transporter and cytochalasin B induce a shift in the emission spectrum peak of band 4.5 to shorter wavelengths by about 1–3 nm. The necessary correction for this shift was less than 1% in 1 mM transporters, and cytochalasin B (assuming no quenching). Changes in the transporters’ absorption spectrum upon addition of ligand were assessed by analysis of the absorption spectra of band 4.5 in the absence and presence of the highest concentrations of ligands added. The difference spectrum of ligand/buffer-transporter-ligand/buffer were virtually identical (in position, shape, and absorbance) to the absorption spectrum of transporter in buffer alone. No correction for absorption spectral changes was considered necessary. Refractive index corrections range from zero to n² (17). The correction for 180 mM D-glucose is ±2%.

Absorption by sugars and cytochalasin B at 295 and 334 nm was barely distinguishable from absorption by buffer alone, hence the systematic errors caused by inner filter effects and fluorescence reabsorption were negligible in studies where the added ligands were sugars and/or cytochalasin B. AMP, ADP, ATP, AMP-CPP, and AMP-PCP at 1 mM absorb weakly at 295 nm (δA 295 = 0.057, 0.057, 0.058, 0.063, and 0.066, respectively) and insignificantly at 334 nm. The nucleotides therefore produce apparent quenching due to attenuation of the exciting light at 295 nm but leave emission at 334 nm unaffected. This inner filter effect was corrected as suggested by Parker (18). The correction takes the form

\[
F_{\text{cor}} = \frac{2.203 A(d2 - d1)}{F_0} \times 10^{-4d} \times 10^{-4d}
\]

where \( A \) is the total absorbance at the excitation wavelength and \( d1 \) and \( d2 \) refer to the geometry of the observed wavelengths. With ATP at 1 mM, this correction results in a 3.4% increase in transporter emission at 334 nm. The transport inhibitor, phloretin, absorbs very strongly both at 295 and 334 nm, hence both inner filter effects and fluorescence reabsorption corrections were necessary. Initially, fluorescence reabsorption by phloretin was corrected by first correcting for inner filter effects, then, after determination of the absorption coefficient of phloretin at each experimental concentration, by applying Lambert’s law to calculate the original intensity of emission. Although sound in principle, this series of corrections could be subject to error due to uncertain geometries. Eventually, a more empirical approach was adopted. The fluorescence of a standard (N-acetyltryptophanamide, NATA) was measured as a function of added ligand (19). After correction for the above systematic errors, the emission of NATA in the presence of all ligands was within 98–100% of control emission (zero ligand) and indicates that NATA does not interact with the ligand employed in this study. Band 4.5 quenching data were then corrected for artifactual quenching by nucleotides and phloretin by running parallel experiments with NATA. The results of corrections using NATA were not significantly different from those obtained by errors alone.

Unless stated otherwise, all data presented in this study have been corrected for artifactual quenching of emission by ligand. Absorption measurements were made at 23 °C using a Beckman DU-8 Spectrophotometer.
quenching (resulting, for example, from absorption of the exciting and emitted light by ligand) or “true” quenching, resulting from interaction of transporter with ligand. Unless stated otherwise, the data presented here represent true quenching of \( E_{\text{obs}} \), produced by ligand/transporter interaction. Procedures for correcting artifactual quenching are described under “Experimental Procedures.”

In the case of cytochalasin B, ligand-induced fluorescence quenching is a simple (one-component) saturable phenomenon and (assuming a significant fraction of the ligand is bound; see Fig. 1a), the dissociation constant for cytochalasin B binding is 0.15 ± 0.03 \( \mu \)M. This value is in good agreement with the \( K_{d} \) for [\(^{3}H\)cytochalasin B binding to transporter (Table I). D-Glucose increases \( K_{d} \) for cytochalasin B-induced quenching (corresponding to the \( K_{d} \) for inhibitor binding) by a factor of 29.4 ± 2.8 (Mean) 34.9 ± 4.7 (Calculated).

**TABLE I**

| Inhibitor | \( [\text{D-Glucose}] \) | \( K_{d} \) | \( K_{d} \) |
|-----------|-----------------|---------|---------|
| Cytochalasin B | 0 | 15.5 | 173 |
| Cytochalasin B | 10 | 14.8 | 158 |
| Cytochalasin B | 30 | 16.8 | 126 |
| Cytochalasin B | 60 | 15.5 | 173 |

Cytochalasin B binding to purified sugar transporter

| Method | Fluorescence |
|--------|-------------|
| \( K_{d} \) | \( B_{\text{max}} \) |
| \( nM \) | \( \mu M \) |
| A | 180 | 143 | 147 | 14.7 |
| B | 142 | 14.8 | 158 | 14.6 |
| C | 167 | 16.8 | 126 | 17.1 |
| D | 128 | 15.5 | 173 | 15.2 |
| E | 149 | 15.2 | 142 | 15.6 |

Molar stoichiometry of binding: 0.84 ± 0.06, 0.85 ± 0.06

Each band 4.5 preparation represents band 4.5 protein purified from a different unit of blood.

**TABLE II**

| Inhibitor | \( [\text{Glucose}] \) | \( K_{d} \) | \( K_{d} \) |
|-----------|-----------------|---------|---------|
| Phloretin | 0 | 0.96 ± 0.08 | 2.05 |
| Phloretin | 0.75 | 1.33 ± 0.12 | 2.3 |
| Phloretin | 2.5 | 1.56 ± 0.14 | 4.3 |
| Phloretin | 5 | 3.30 ± 0.19 | 2.05 |

Effect of D-glucose on inhibitor-induced fluorescence quenching

These measurements were made as in Fig. 1, a and b.

- \( K_{d} \) for inhibitor binding was obtained from the reciprocal of the slope of the plot 1/(1 - R) versus [CCB] (where R is the measured \( K_{a} \) of the inhibitor concentration) in cytochalasin B experiments and as reciprocal of the slope of an Eadie-Scatchard plot in phloretin experiments.

- \( K_{d} \) for D-glucose inhibition of inhibitor binding was calculated assuming simple competitive inhibition, i.e. \( [\text{glucose}] / K_{d} = (R / K_{d} / [\text{glucose}]) - 1 \) where \( K_{d} \) is the measured \( K_{d} \) in the absence of sugar. The assumption of competitive inhibition was confirmed by plots of \( [\text{glucose}] / K_{d} \) versus \( [\text{glucose}] / K_{d} \). Such graphical analysis of both cytochalasin B and phloretin data produced straight line plots (correlation coefficient > 0.99 in each instance).

- Observed \( x \) intercept, taken as \( K_{d} \).

Fig. 1. Effects of cytochalasin B (CCB) and D-glucose (Glc) on intrinsic fluorescence of the purified red cell sugar transporter. Analysis of CCB-induced fluorescence quenching is shown in a. CCB was added as successive amounts (0.025–100 \( \mu \)M) of stock solution to 0.1 (A), 0.2 (B), and 0.4 (C) \( \mu \)M solutions of transporter. Above 20 \( \mu \)M, further addition of CCB was without effect on the fluorescence signal. Fractional saturation, \( R \), was calculated as change in emission upon addition of CCB divided by maximum emission change observed (i.e. upon addition of 20–100 \( \mu \)M CCB). A plot of 1/(1 - R) versus [CCB] has a slope of 1/\( K_{d} \) and \( x \) intercept for cytochalasin B binding site concentration. The calculated \( K_{d} \) values for A, B, and C are 0.14 ± 0.01, 0.17 ± 0.02, and 0.17 ± 0.02 \( \mu \)M, respectively. In B, Eadie-Scatchard analysis of phloretin-induced band 4.5 fluorescence quenching is shown. Ordinate, -\( \triangle E \)/[phloretin] (\( \triangle E \) = change in emission/phloretin concentration in \( \mu \)M); abscissa, -\( \triangle E \). Fluorescence in the absence of phloretin was adjusted to 100 units. This plot takes the form of -\( \triangle E \)/[phloretin] versus -\( \triangle E \). The slope of the obtained line is -1/\( K_{d} \), giving a value for \( K_{d} \) of 0.96 ± 0.08 \( \mu \)M. Number of determinations per point, 4 or more. In c, concentration-dependence of D- (○) and L- (●) glucose-induced band 4.5 fluorescence quenching is shown. Successive amounts (0.5–100 \( \mu \)M) of stock glucose solutions were added to a 0.2 \( \mu \)M solution of transporter. These data have been corrected for dilution effects only. Fluorescence in the absence of glucose was 1590 units. In d, Eadie-Scatchard analysis of D-glucose-induced fluorescence quenching. The \( K_{d} \) for D-glucose binding is 0.15 ± 0.03 \( \mu \)M.
B-induced band 4.5 fluorescence quenching, respectively. This suggests that band 4.5 contains both the cytochalasin B- and phloretin-binding sites detected in intact cell transport studies (28). Moreover, asymmetry in D-glucose binding to the transporter appears to be an intrinsic property of the system (24). In intact cells, phloretin binds to an exofacial site on the sugar transport protein and cytochalasin B to an intracellular site (29). As competitions between phloretin and D-glucose and between cytochalasin B and D-glucose are mediated by the low and high Kd D-glucose-binding sites, respectively, these data suggest that the low Kd substrate-binding site is associated with the external (influx) orientation of native transporter and the high Kd site with the internal (efflux) orientation of native transporter. Two-component saturable quenching was also observed using 4,6-ethylidene glucose (see Fig. 3a), a non-transported D-glucose analogue that binds to the extracellular substrate-binding site of the transporter with high affinity and to the intracellular site with much lower affinity (28). Other sugars known to act as substrate for the red cell hexose transport system also induce two-component quenching (Table III). Two components of sugar-induced fluorescence quenching were not observed in a previous study. However, the lowest substrate concentration employed in this study (21) was in the order of 10 mM, at which concentration the low Kd binding sites would be 85-90% saturated with sugars and, therefore, undetectable. It should be emphasized that as the low Kd is detected under conditions where dilution artifacts are minimal and substrate concentration are lowest, those systematic errors that influence steady-state fluorescence measurements (see above) are less likely to influence the measurements than under conditions (high ligand concentrations and greatest dilution factors) where the high Kd is detected.

Addition of ATP to purified transporter results in significant fluorescence quenching of the protein (see Fig. 3a). Quenching is complete within 20 s (data not shown). Two lines of evidence suggest that ATP-induced fluorescence quenching is the result of specific ligand binding and not external quenching by nucleotide. 1) AMP, ADP, and ATP share almost identical absorption spectra (see "Experimental Procedures") yet quenching produced by ATP is considerably less than that produced by either AMP or ADP (Fig. 3). Fig. 2b shows that uncorrected quenching produced by AMP and ADP is a linear function in nucleotide concentration. Uncorrected quenching by ATP consists of this linear component superimposed upon a saturable component of quenching (Kd app = 53 μM; see Fig. 2b). Correction of these nucleotide

**Table III**

**Components of sugar-induced transporter fluorescence quenching**

Results are shown as mean ± 1 S.D. These results are obtained by first assuming that the marked curvature of Eadie-Scatchard plots of quenching data results from two components of substrate-induced quenching, followed by calculation of two Kd app parameters by the method of successive approximation (27).

| Sugar       | Low Kd app | High Kd app | n  |
|-------------|------------|-------------|----|
| L-Glucose  | N.D.       | N.D.        | 5  |
| D-Glucose  | 1.5 ± 0.2  | 26.9 ± 3.2  | 7  |
| Galactose  | 7.4 ± 0.5  | 84.3 ± 6.9  | 3  |
| 2-Deoxy-D-glucose | 1.5 ± 0.2 | 37.4 ± 2.2 | 3  |
| Ethylidene glucose | 1.9 ± 0.3 | 29.7 ± 3.4 | 6  |
| Maltose    | 2.6 ± 0.1  | 128 ± 11.4  | 3  |

* L-Glucose does not interact significantly with the erythrocyte hexose transport system.

+ N.D., not detectable.

+ Sugars that interact with but, for steric reasons, are not transported by the erythrocyte hexose transport system.

Data for the inner filter effect results in the loss of the linear component of quenching induced by ATP alone with Kd app of 51 μM. These considerations indicate that ATP-induced quenching is neither artifactual nor mediated solely via interaction of the transporter with the ribose moiety of the nucleotide. 2) Triphosphopyridinenucleoside pentosadial (10 μM–1 mM) is without significant effect on Kd app (Fig. 2b). These data indicate that quenching is ATP-specific and is not mediated solely by interaction of positively

**Fig. 2. Effect of ATP, ADP, and AMP on intrinsic fluorescence of transporter.** In a, fluorescence emission spectra of transporter (0.4 μM) in the absence (A) and presence of 3 mM AMP (B) and ATP (C), pH 7.0, are shown. Curves D shows the emission spectrum for ATP and/or AMP medium in the absence of transporter. These data are not corrected for the inner filter effect. b shows the concentration dependence of nucleotide-induced fluorescence quenching. These data are corrected only for dilution effects. Fluorescence in the absence of added ligand was normalized prior to each experiment to 190 units. The curve shown through the ATP data correspond to a section of a single rectangular hyperbola (Kd = 53 ± 4 μM) superimposed on the linear component of quenching observed in the presence of AMP and ADP. When corrected for inner filter effect, the linear component of nucleotide-induced quenching is lost (B). The effects of tripolyphosphate pentasodium on transporter fluorescence are also indicated (V). Number of determinations per point, 4 or more. c shows the analysis of ATP-induced fluorescence quenching. ATP was added as successive amounts (0.05–90 μM) of stock (5 mM, pH 7.0) to a 0.22 mM solution of transporter (pH 7.0). Nonspecific quenching and dilution effects were corrected by running parallel experiments using AMP. Above 5 mM, further addition of ATP is without effect on the fluorescence signal. Fractional saturation, R, was calculated as change in emission upon addition of ATP divided by maximum emission change observed (i.e., upon addition of 5 mM ATP). A plot of 1/(1 − R) versus [ATP]/R shows a slope of 1/Kd and an intercept of total ATP-binding site concentration. The y and x scales are expanded close to the origin to indicate the x intercept. Regression analysis results in a Kd of 50 ± 0.9 μM and a concentration of ATP-binding sites of 0.188 ± 0.015 μM (the arrows indicate the S.D. of the calculated y intercept). This corresponds to a binding site:transporter molar ratio of 0.87 ± 0.08 and an ATP:cytochalasin B-binding site molar ratio of 1.01 ± 0.03. Number of separate determinations per point, 4 or more.
charged transporter domains with polyanionic species. Addition of 6 mM MgCl₂ is without effect on the kinetics of nucleotide-induced fluorescence quenching (Table IV). The stoichiometry of ATP interaction with band 4.5 can be assessed indirectly from fluorescence quenching data using the procedures employed for analysis of the stoichiometry of cytochalasin B binding to band 4.5 (see Fig. 1a). Fig. 2c shows such an analysis. Here, the linear component of quenching produced by ATP was subtracted from the total quenching by subtracting AMP-induced quenching from ATP-induced quenching data. Band 4.5 protein was present as 25 µg in 2.1 ml. Over the range 50 nM–90 µM ATP, the extrapolated concentration of ATP binding sites was 0.188 ± 0.013 µM. Assuming an average molecular size of 55 kDa for band 4.5 protein, this represents an ATP to band 4.5 binding ratio of 0.87 ± 0.06:1, a result indistinguishable from the ratio of cytochalasin B-binding sites per band 4.5 protein (Fig. 1a) and assuming each cytochalasin B-binding site represents a functional transporter molecule, this result suggests that each transporter protein contains a single ATP binding/interaction site. Direct binding studies using labeled ATP are necessary to confirm this point. AMP-CP and AMP-CP (metabolizable and non-metabolizable ATP analogues, respectively) both appear to share ATP's ability to induce quenching but with 5-fold lower affinity (Kd = 230–250 µM) and 5-fold lower maximum quenching (data not shown). The magnitude of fluorescence changes is low (2–3% following correction) and the calculated quenching parameters are unlikely to be as reliable as those obtained using ATP.

ATP modifies the ability of substrate to induce fluorescence quenching of the transporter. Fig. 3A shows that ATP (500 µM) reduces the low Kd for Eglc-induced fluorescence quenching from 2.16 ± 0.21 to 0.38 ± 0.04 mM (Fig. 3A). The high Kd for Eglc is increased slightly from 46.2 ± 1.8 to 55.6 ± 1.4 mM. Similar results were obtained using D-glucose (Fig. 3B). These effects of ATP are half-maximal at about 45 µM ATP (Fig. 3A) and are unaffected by the addition of 6 mM MgCl₂. The non-metabolizable and metabolizable ATP analogues (AMP-CP and AMP-CP (metabolizable and non-metabolizable ATP analogues, respectively)) both appear to share ATP's ability to induce quenching but with 5-fold lower affinity (Kd = 230–250 µM) and 5-fold lower maximum quenching (data not shown). The magnitude of fluorescence changes is low (2–3% following correction) and the calculated quenching parameters are unlikely to be as reliable as those obtained using ATP.

**Table IV**

Effects of MgCl₂ on ATP regulation of glucose transporter

The measured parameters were KappATP, Kapp for ATP-induced quenching; KappK₆, the Kapp for ATP-induced reduction in the low Kd for glucose binding to transporter; KappK₆, the Kapp for ATP-induced increase in the high Kd for d-glucose binding to the transporter; KappK₆, that concentration of ATP that half-maximally reduces K₆ for d-glucose efflux from IOVs; KappVmax that concentration of ATP that half-maximally reduces Vmax for d-glucose efflux from IOVs.

| Measured parameter | [MgCl₂] | 0 | n | 6 | n |
|--------------------|---------|---|---|---|---|
| KappATP µM         |         |   |   |   |   |
| KappK₆ µM          |         |   |   |   |   |
| KappK₆ µM          |         |   |   |   |   |
| KappVmax µM        |         |   |   |   |   |

**Fig. 3. Effect of ATP on ligand-induced fluorescence quenching.** A shows the effect of ATP (300 µM) on Eglc-induced fluorescence quenching. Ordinate, change in emission/[Eglc]; abscissa, change in emission. EGlC concentrations ranged from 0.5 to 180 mM. Protein concentration, 0.2 µM. Successive approximation analysis of the two-component Eadie-Scatchard plots results in K₆ for Eglc of 2.16 ± 0.21 and 46.2 ± 1.8 mM (0 ATP) and 0.38 ± 0.04 and 55.6 ± 1.4 mM (500 µM ATP). B shows the effect of ATP (0.5 mM) on D-glucose-induced fluorescence quenching of transporter. Ordinate and abscissa are as in A. Successive approximation analysis of the two-component Eadie-Scatchard plots results in K₆ for Glc of 1.33 ± 0.21 and 25.2 ± 1.8 mM (0 ATP) and 0.61 ± 0.04 and 103.6 ± 5.6 mM (500 µM ATP). In ATP experiments, sugar was added in successive amounts (from 1 mM stock sugar, 0.5 mM ATP, pH 7.0) to a 0.2 µM solution of transporter in 0.5 mM ATP (pH 7.0).

sugar-free medium in efflux experiments (29, 30). In this current study, 5 µM cytochalasin B reduced the rate of d-glucose efflux from IOVs by more than 99% (see Fig. 4). The measured permeability coefficient (Pₛ) for d-glucose efflux from IOVs in the presence of cytochalasin B is 7.8 ± 2.8 × 10⁻⁵ cm/s (n = 23; 20 °C) whereas Pₛ for unrestricted efflux (efflux in the absence of cytochalasin B; Vmax/K₆, (30)) is 1.35 ± 0.12 × 10⁻⁵ cm/s (n = 19). These values are similar to those reported for extensively washed red-cell ghosts (8, 30, 31, 32).

As reported previously (8), efflux from IOVs resembles both efflux and influx in red cell ghosts but not influx in intact red cells. In terms of Michaelis-Menten parameters, Vmax and K₆ for cytochalasin B-inhibitable d-glucose efflux from IOVs (and influx in ghosts) are some 4–5-fold greater than Vmax and K₆ for influx in intact cells. However, if efflux from IOVs is monitored into red cell lysate (obtained by lysis of cells), the rate of glucose exit is reduced and K₆ and Vmax are reduced to values resembling K₆ and Vmax for influx into intact cells (8). Fig. 4 shows that ATP mimics the ability of red cell lysate to reduce the rate of d-glucose exit from IOVs. Analysis of these time course data using the integrated Michaelis-Menten
FIG. 4. Effects of ATP (0.5 mM) and cytochalasin B (CCB, 5 μM) on the time course of d-glucose exit from IOVs. Ordinates, fraction of intravesicular glucose remaining within the IOVs; abscissa, time in seconds. The curves to the right (experimental) are tracings of chart recordings of turbidimetric measurements of d-glucose exit from IOVs pretreated for 30 min with 0.5 mM ATP (lower records) or 0 ATP (upper records). Efflux from ATP-treated IOVs was measured into Tris medium containing 0.5 mM ATP, pH 7.0. For both conditions, exit was measured in the presence (+CCB) and absence (−CCB) of 5 μM CCB. The recordings of exit in the absence of CCB are extrapolated (-----) to zero time, K_m and V_max for exit in the absence of CCB (calculated as described under “Experimental Procedures”) were 14.5 mM and 1.6 mmol/l of water/s, respectively, in the absence of ATP and 4 mM and 0.7 mmol/l of water/s, respectively, in the presence of ATP. The curves to the left (predicted) are calculated for control (0 ATP, 0 CCB, upper curve) and ATP-treated IOVs (0 CCB, lower curve) from the experimental K_m and V_max parameters. The equation describing the time course of exit is

\[ P \cdot \frac{K_m}{P + C} \ln \left( \frac{S_i}{S_f} \right) + \frac{(P + C + K_m)}{(P + C)} (S_i - S_f) = V_{\text{max}} \cdot t \]

where C is the concentration of d-glucose within the IOVs at zero time (60 mM). The remaining symbols are described under “Experimental Procedures.” S_i was solved by computer using an iterative calculation procedure. Temperature, 20 °C.

rate equation reveals that ATP reduces K_m and V_max for cytochalasin B-inhibitable d-glucose efflux from IOVs (Fig. 5B). This effect of ATP is half-maximal at about 44 μM ATP. Addition of 6 mM MgCl_2 is without effect on the kinetics of ATP modulation of transport (Table IV). AMP, ADP, AMP-CPP, and AMP-PCP are unable to mimic the ability of ATP to reduce K_m and V_max for efflux from IOVs (Fig. 5C). ATP was without effect on cytochalasin B-insensitive d-glucose efflux (leakage) from IOVs (Fig. 4). As with the effects of cell lysate on sugar efflux from IOVs (8), the effects of ATP (0.5 mM) on K_m for d-glucose exit from IOVs are seen immediately upon mixing IOVs and ATP (Table V) and are reversed immediately upon assay of d-glucose exit from ATP-treated IOVs into ATP-free medium (Table V). Effects of ATP on V_max for exit from IOVs are expressed more slowly and are not reversed within 30 min of incubation of ATP-treated IOVs in ATP-free medium (Table V).

DISCUSSION

This study demonstrates that ATP modifies the ability of human red cell membrane IOVs to transport d-glucose. This effect is not brought about by nucleotide-induced alterations in nonspecific transmembrane d-glucose flux (leakage) for cytochalasin B, a potent inhibitor of the catalytic activity of the red cell sugar transport protein (29, 30), reduces the permeability of IOVs to d-glucose 2000-fold, leaving a residual flux (a leakage of less than 0.1% of control flux) that is unaffected by ATP. Moreover, ATP modifies the ability of d-glucose to induce quenching of the intrinsic tryptophan fluorescence of purified sugar transport protein. In both instances, these effects are half-maximal at about 50 μM ATP, and indirect analysis suggests a probable stoichiometry of ATP transport protein interaction of 1:1. These findings establish that the catalytic properties of the red cell sugar transporter are subject to modulation by ATP and suggest the potential

FIG. 5. Analysis of the ATP dependence of altered transporter affinity for substrate and effects on transport in inside-out vesicles. Effect of ATP on low K_app (K_a) data indicated by the open circles) and high K_app (K_a) data indicated by the filled circles) for d-glucose-induced quenching of purified transporter fluorescence are shown in A. Ordinate, K_a: μM; abscissa, ATP concentration in μM. These results summarize 21 separate experiments. Transporter concentration was 0.2 μM in 1, 25, 50, 100, 200, 325, and 500 μM ATP. K_a and K_2 were calculated by the method of successive approximation. The curves drawn through the points are sections of rectangular hyperbolae with K_app of 44 ± 2 μM (for K_a data) and 47 ± 3 μM for K_2 data. Effect of ATP on K_a and V_max cytochalasin B-inhibitable d-glucose efflux from human red cell inside-out vesicles is shown in B. Ordinate: K_a, mM (•); V_max, mmol/l of IOV water/s (△); abscissa, ATP concentration. Glucose-loaded (60 mM) IOVs were incubated on ice in 60 mM d-glucose Tris medium containing the appropriate ATP concentration (0, 25, 50, 75, 250, or 500 mM) for 30 min prior to injection into sugar-free Tris medium containing ATP (0, 25, 50, 75, 250, or 500 μM). Each point represents the mean ± 1 S.D. of at least 7 separate experiments. Glucose efflux in the presence of 5 μM cytochalasin B was 0.7 ± 0.01 mmol/l of IOV water/s (n = 23; some 2000-fold lower than control efflux) and was unaffected by ATP. The curves drawn through the points are single sections of rectangular hyperbolae calculated by a nonlinear regression fit to the data with K_app of 44.9 ± 4.9 μM (K_a data) and 39.9 ± 3.1 μM (V_max data). In C, effect of various nucleotides at 1 mM on K_a and V_max cytochalasin B-inhibitable d-glucose efflux from IOVs is shown. Experimental conditions are as in 4B. Ordinate: K_a, mM (open bars); V_max, mmol/l of IOV water/s (hashed bars). The added nucleotides are indicated below the bars. Results are shown as mean ± 1 S.D. Number of experiments, 4.
for a mechanism of sugar transport regulation in cells that is fundamentally different to the well-documented, insulin-dependent recruitment of intracellular hexose transporter to plasma membrane (1-4). As these effects are observed in the human red cell, such a mechanism must necessarily involve modification of the catalytic properties of transport proteins located within the plasma membrane.

Although it has been suggested that a higher molecular weight protein and not band 4.5 may be the native sugar transport protein (33), this current study shows that band 4.5 protein contains the transport substrate-binding sites (exposed at endo- and exofacial orientations of the transporter), the normally endofacial cytochalasin B-binding site, and an ATP-binding site. These sites are integral components of the red cell sugar transport system (28, see also above). In addition, it is known that reconstitution of band 4.5 into artificial membranes results in the reconstitution of sugar transport activity with kinetic properties (Michaelis constant and turnover numbers, exchange fluxes) that closely resemble the properties of native sugar transport (24). These findings further strengthen the view that band 4.5 protein contains the red cell sugar transporter (12).

Sugar transport in intact human red cells displays catalytic asymmetry (24, 28, 30). \( V_{\text{max}} \) and \( K_m \) for glucose influx are some 5-10-fold lower than the corresponding values for efflux (28). It has been suggested that the complexation of cell water and d-glucose by hemoglobin could result in apparent sugar transport asymmetry in red cells (28). Transport asymmetry is reduced or even lost in red cell ghosts (10, 30-32). The net effect is increased \( V_{\text{max}} \) and \( K_m \) for sugar entry and reduced \( K_m \) for sugar exit (8). This is a reversible phenomenon. Resynthesis of ghosts followed by resealing in the presence of high concentrations of cell lysate restores transport asymmetry (8). These effects seem not to be brought about by the presence of hemoglobin in cell lysate but rather by the presence of low molecular size factors (<10 kDa) (8). This present study supports the view that ATP may be such a factor, because the nucleotide mimics the ability of cell lysate to reduce both \( K_m \) and \( V_{\text{max}} \) for d-glucose efflux from IOVs. Half-maximal inhibition of sugar efflux from IOVs by cell lysate was produced at endofacial cytochalasin B-binding site, the normally exofacial phloretin-binding site, and an ATP-binding site sugar transport molecule or whether the occupation of one site by substrate precedes the occupation of the other site by substrate. This is an important question because to date, one-sugar transport models have been uniformly unsuccessful in predicting the complex kinetic features of human red cell sugar transport (28). The one-site model (with respect to sugar-induced fluorescence quenching) may be described by the scheme shown below.

\[
\begin{align*}
XG & \xrightarrow{K_a} G + X + G \xrightarrow{K_b} XG_g \\
& \xrightarrow{\beta q} \xrightarrow{\alpha q}
\end{align*}
\]

where \( X \) is the transporter which may only bind a single sugar at either an internal or external site at any point in time, and \( \beta q \) refers to quenching produced by the \( XG \), and \( XG \), complexes, respectively. For a non-transported sugar (e.g. EGlc or maltose), sugar-induced quenching, \( q \) is given by

\[
q = \frac{Q_{\text{m}}[S][\alpha + \beta K_a/K_b]}{K_a(1 + [S]/K_a) + [S]}
\]

where \( Q_{\text{m}} \) is maximum theoretical quenching and \( S \) replaces \( G \) and \( G \) (the protein/lipid particles are freely permeable to sugar). Substituting values for \( K_a \) and \( K_b \) (1 and 25 mM) and varying \( \alpha \) and \( \beta \) parameters results in a series of parallel lines in Eadie-Scatchard plots with no indication of two-component quenching kinetics. The two-site model, where two sugars can bind simultaneously to the carrier, is shown below.

\[
\begin{align*}
XG & \xrightarrow{K_a} G + X + G \xrightarrow{K_b} XG_g \\
& \xrightarrow{\beta q} \xrightarrow{\alpha q}
\end{align*}
\]

Here, the transporter \( X \) can bind sugars simultaneously at internal and external sites. \( \beta q \) and \( \gamma q \) refer to quenching produced by \( XG \), \( XG \), and \( XG \), complexes, respectively. For a non-transported sugar, sugar-induced quenching, \( q \), is given by the following.

\[
q = \frac{Q_{\text{m}}[S][\alpha + \beta K_a/K_b + \gamma[S]/K_a]}{K_a(1 + [S]/K_a) + [S](1 + [S]/\gamma K_a)}
\]

Substituting values for \( K_a \) and \( K_b \) and varying \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) parameters results in a series of plots in Eadie-Scatchard analyses, some with no indication (e.g. when \( \alpha = \beta = \gamma = \delta = 1 \)) and others with clear indications of multi-component quenching. Multi-component quenching is predicted both by a system of two identical binding sites (\( K_a = K_b \) with strong

### Table V

**Time course of ATP action on sugar efflux from IOVs**

| Preincubation in ATP* | Into ATP-free Medium | Into ATP medium* |
|-----------------------|----------------------|------------------|
| \( K_m \) | \( V_{\text{max}} \) | \( K_m \) | \( V_{\text{max}} \) |
| \( \text{min} \) | \( \text{mM} \) | \( \text{mmol/l IOV water/min} \) | \( \text{mM} \) | \( \text{mmol/l IOV water/min} \) |
| 0 | 13.2 ± 1.6 | 180 ± 5 | 2.7 ± 0.3 | 98 ± 4 |
| 5 | 11.6 ± 1.3 | 102 ± 6 | 3.4 ± 0.4 | 67 ± 5 |
| 15 | 12.9 ± 1.4 | 68 ± 3 | 3.9 ± 0.2 | 45 ± 4 |
| 30 | 10.2 ± 0.8 | 45 ± 7 | 2.4 ± 0.2 | 42 ± 3 |
| 30* | 11.7 ± 0.5 | 72 ± 4 | 3.1 ± 0.1 | 56 ± 2 |

*0.5 mM ATP.

* Incubated in ATP medium for 30 min followed by incubation in ATP-free medium for 30 min at 20 °C. Efflux measured at 20 °C.
negative cooperativity ($\theta > 1$) and by a system of two different binding sites with insignificant interaction ($\theta = 1$; $K_a < K_b$). With the former, the fluorescence quenching produced by formation of the binary and ternary complexes of $X, G$, and $G_i$ can be identical. With the latter system, quenching produced by formation of the ternary complex must be greater than that produced by formation of the binary complex of $X$ and $G_i$. It is not possible, using the available experimental data, to distinguish between these possibilities. We may conclude, however, that the one-site model is inconsistent with the experimental data.

This present study does not support the view that the transporter is of the one-site type. However, two previous studies have provided evidence in favor of the one-site model (29, 35). The study of Krupka and Deves (29) modeled inhibition of red cell sugar transport (mediated by both one- and two-site kinetic schemes) produced by phloretin and cytochalasin B. They demonstrated that transport was consistent with the one-site model. They also described conditions under which their conclusions would be invalidated. These conditions require that phloretin and cytochalasin B cannot bind to the transporter simultaneously and that binding of sugar to one of the two sites on the two-site carrier does not prevent inhibitor or sugar binding to the second. It has previously been shown that phloretin inhibits cytochalasin B binding to purified transporter (36), and this present study supports the view that binding of sugar to one binding site does not prevent binding of inhibitor or sugar to the second. The criteria necessary to reject their conclusion of one-site kinetics are, therefore, fulfilled. Gorga and Lienhard (35) adopted a different approach to this problem. Measuring cytochalasin B binding to red cell membranes stripped of peripheral proteins they found that ethylidene glucose (EGlc—a non-transported sugar that binds preferentially to the interior or orientation of the transporter) competitively inhibited binding with a $K_{iapp}$ of 26 mM. They concluded that EGlc can displace CCB binding to the internal site by binding to the external orientation of the one-site transporter. In this study, it is shown that two EGlc-binding sites are detected in band 4.5—one with a $K_{dapp}$ of 2 mM and a second with $K_{dapp}$ of 30 mM. In red cell membranes stripped of peripheral proteins, these values are 1 and 28 mM (34). It is possible, therefore, that the observed EGlc-inhibition of CCB binding to the transporter was mediated via the high $K_{dapp}$ binding site. The high affinity, low $K_{dapp}$ site was without effect on CCB binding. In support of this possibility, the predictions of a two-site model in which $G_i$ and CCB compete for binding to an internal site but in which $G_i$ and CCB do not compete for binding have been derived. The basic scheme is shown below.

Table VI: Effects of sugars on cytochalasin B binding to red cell ghosts stripped of peripheral membrane proteins

| [Sugar] | Bound/free | Measured* | Calculated* |
|---------|------------|-----------|-------------|
| Propylglucoside | EGlc |          |             |
| mM       |       |           |             |
| 0        | 0      | 1.34 ± 0.54 | 4.95        |
| 40       | 0      | 5.55 ± 0.05 | 3.14        |
| 80       | 0      | 3.44 ± 0.27 | 1.62        |
| 160      | 0      | 1.64 ± 0.11 | 3.84        |
| 0        | 40     | 3.7 ± 0.23  | 2.98        |
| 6        | 80     | 2.12 ± 0.19 | 1.35        |
| 0        | 150    | 1.18 ± 0.02 |             |
| 40       | 40     | 2.34 ± 0.09 |             |

* Taken from Gorga and Lienhard (35).

** Calculated as described under "Discussion."

Calculated assuming a $K_a$ for binding of a mixture of propylglucoside and EGlc of 27.5 mM.
simple one-site model is inconsistent with the available transport and fluorescence quenching data, there is no stringent requirement to assume that $K_f$ for inhibition of transport by extracellular EGlc $\approx K_{app}$. On the other hand, $K_f$ for inhibition of exchange fluxes mediated by an asymmetric, two-site transport system by extracellular EGlc ($K_{app}/6$) is in the order of 1.2–1.8 mM, a value very close to the low $K_{app}$ for EGlc-induced transporter fluorescence quenching at 23 °C ($1.9 \pm 0.3$ mM).

The major enigma surrounding red cell transport centers upon the observation of two Michaelis constants for sugar exit but only one for sugar entry (28). One-site transport models cannot account for these features of sugar transport. It has been suggested that the problem lies not with the models but rather with the experimental determination of sugar transport kinetics (24, 35). Specifically, it has been proposed that previous transport determinations are in error, possibly due to the differential transport of $\alpha$- and $\beta$-glucose by the human red cell (24, 35). A number of direct, experimental observations argue against this hypothesis. 1) The predicted multi-component washout of d-glucose from loaded red cells due to the differential transport of d-glucose anomers is not observed experimentally (39). 2) $\alpha$- and $\beta$-glucose are transported identically by the intact human red cell (22). 3) Human red cell sugar transport complexity (two Michaelis constants for exit and one for entry) is lost in red cell ghosts (8) and may be restored upon reincorporation of red cell lysate (8) or ATP (34) into ghosts. 4) Previous sugar transport determinations made in a variety of laboratories using a variety of experimental techniques and procedures are in substantial agreement (28). It appears, therefore, that human red cell sugar transport complexity is a real phenomenon that may, in some way, be due to modulation of the intrinsic properties of the transporter by factors (possibly ATP) present in red cell cytosol. In addition, the inability of one-site transport models to account for human red cell sugar transport is sufficient to reject their use in kinetic descriptions of the transfer mechanism.

A two-site transport model for human red cell sugar transport has been proposed (34) in which ATP interacts with the transporter (in as yet unspecified fashion). This model predicts the following properties of the transport system. 1) Asymmetry in $V_{max}$ and $K_m$ parameters for sugar entry and exit in fresh cells and ATP-loaded ghosts; 2) the presence of two Michaelis parameters for sugar exit and one for entry in fresh cells and ATP-loaded ghosts; 3) Transport symmetry in ATP-free ghosts (only one Michaelis parameter for exit and entry); 4) increased $K_m$ for equilibrium-exchange d-glucose transport in ATP depleted red cells. This model is consistent with both available transport data and ligand binding data. However, the transport system is not of the rapid equilibrium type. As the $K_{app}$ parameters for sugar binding to internal and external orientations of the transporter are widely different (see above), yet $K_m$ and $V_{max}$ for influx and efflux in ghosts are identical, we must conclude that the Michaelis and velocity constants measured in transport determinations are the product of both substrate binding and translocation constants (e.g. see Ref. 40). Such a model requires steady-state analysis and is of the Hybrid Ping Pong Random type (40) with a central Random Bi Bi segment to account for exchange fluxes and two Ordered Uni Uni segments to account for the partially reactive zero-trans fluxes (transport in the absence of sugar at the opposite side of the membrane). In addition, steps must be included to account for the effects of ATP on transport. ATP acts to increase $K_m$ for exit, reduce $K_m$ and $V_{max}$ for entry, and to reduce $K_m$ for transport under exchange conditions.

What is the molecular mechanism of ATP action of sugar transport? Although ATP modulates the properties of purified transporter with almost identical potency to its modulation of sugar transport in IOVs, it is not yet possible to ascertain whether ATP modification of transport reflects direct transporter/nucleotide interaction or whether intermediate species (e.g. membrane and cytoskeletal kinases) are also involved. Certainly, purified transporter is not phosphorylated by ATP in the absence of exogenous kinase but is capable of acting as substrate for protein kinase C (41). In addition, the transporter is phosphorylated in vivo in the absence of phorbol ester and exogenous protein kinase C (41). In view of the inability of AMP and ADP to mimic ATP inhibition of sugar transport in IOVs, the rapid onset and reversibility of ATP action on $K_m$ for transport and the somewhat slower onset and delayed recovery of ATP action on $V_{max}$ for transport, it is interesting to speculate that phosphorylation of the transporter results in reduced catalytic activity ($V_{max}$) and that simple allosteric interaction with ATP modulates $K_m$ for transport (see Randle and Smith (5)). In the absence of additional experimental evidence, the elucidation of the mechanism of this recruitment-independent form of sugar transport regulation must await further study. Although the red cell may never encounter physiological conditions in which intracellular ATP levels fall below 50 $\mu$M, these findings may be of physiological significance in muscle and avian erythrocytes where, as with the human red cell, ATP depletion results in insulin-independent sugar transport stimulation (5, 6, 42).

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