J. Membrane Biol. 132, 167–178 (1993)

The Journal of
Membrane Biology

Initial Steps of α- and β-D-Glucose Binding to Intact Red Cell Membrane

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Summary. The kinetics of the initial phases of D-glucose binding to the glucose transport protein (GLUT1) of the human red cell can be followed by stopped-flow measurements of the time course of tryptophan (trp) fluorescence enhancement. A number of control experiments have shown that the trp fluorescence kinetics are the result of conformational changes in GLUT1. One shows that nontransportable L-glucose has no kinetic response, in contrast to D-glucose kinetics. Other controls show that D-glucose binding is inhibited by cytochalasin B and by extracellular D-maltose. A typical time course for a transportable sugar, such as D-glucose, consists of a zero-time displacement, too fast for us to measure, followed by three rapid reactions whose exponential time courses have rate constants of 0.5–100 sec^{-1} at 20°C. It is suggested that the zero-time displacement represents the initial bimolecular ligand/GLUT1 association. Exponential 1 appears to be located at, or near, the external membrane face where it is involved in discriminating among the sugars. Exponential 3 is apparently controlled by events at the cytosolic face. Trp kinetics distinguish the K_d of the epimer, D-galactose, from the K_d for D-glucose, with results in agreement with determinations by other methods. Trp kinetics distinguish between the binding of the α- and β-D-glucose anomers. The exponential 1 activation energy of the β-anomer, 13.6 ± 1.4 kcal mol^{-1}, is less than that of α-D-glucose, 18.4 ± 0.8 kcal mol^{-1}, and the two Arrhenius lines cross at ≈23.5°C. The temperature dependence of the kinetic response following α-D-glucose binding illustrates the interplay among the exponentials and the increasing dominance of exponential 2 as the temperature increases from 22.3 to 36.6°C. The existence of these interrelations means that previously acceptable approximations in simplified reaction schemes for sugar transport will now have to be justified on a point-to-point basis.

Key Words red cell · glucose transport protein · GLUT1 · kinetics · rapid reactions · tryptophan

Introduction

The conformational change that follows D-glucose binding (Krupka, 1971) to the human red cell glucose transport protein (GLUT1) has been observed in the purified glucose transporter by Gorga and Lienhard (1982), who monitored the D-glucose-induced quenching of tryptophan (trp) fluorescence. Subsequently, Appleman and Lienhard (1985) used rapid reaction methods and trp fluorescence kinetics to determine that the time constant for the initial binding of 4,6-O-ethylidene-D-glucose to the purified glucose transporter was in the 10-msec range. The combination of rapid reaction methods with measurements of trp fluorescence enhancement has made it possible for us to obtain continuous data covering the initial phases of D-glucose binding to GLUT1 in fresh red cells. The time resolution of our stopped-flow spectrofluorimeter is fast enough to permit the deconvolution of three exponential reactions during the initial steps of D-glucose binding to GLUT1, with time constants ranging from 6 msec to 1 sec. In addition, there is an initial zero-time displacement in trp fluorescence, whose time constant is too fast for us to resolve, and which we have tentatively assigned to the bimolecular association of sugar with GLUT1. Data obtained from the kinetics of D-glucose binding show that D-glucose binds more tightly than D-galactose, in accordance with the view that H-bonding to the -OH group at C-4 is an important determinant of D-glucose binding, as suggested by Kahlenberg and Dolansky (1972) and Barnett, Holman and Munday (1973). Our observation that the activation energy for binding of the β-D-glucose anomer is lower than that for the α-anomer confirms the importance of the H-bond to β-D-glucose in the GLUT1 binding process (Barnett et al., 1973).

The detailed time dependence of the fluorescence enhancement, even for a single anomer, is dominated by different exponential terms at different temperatures, which shows that all three of the exponential terms are important in sugar binding. Thus, it is difficult to justify neglect of any of the reaction steps, so that simplifications that have previously been used to make the kinetic analysis mathematically tractable will now require justification on a point-to-point basis.

Materials and Methods

Materials

α-D-glucose, D-maltose and 4,6-O-ethylidene-D-glucose were obtained from Aldrich Chemical (Milwaukee, WI). β-D-glucose, L-glucose (mixed anomers), D-galactose, adenosine triphosphate (ATP), and cytochalasins B (Cyt B) and E (Cyt E) were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were also of reagent grade and obtained from Sigma or Fisher Scientific (Springfield, NJ).

Methods

Fresh human blood was drawn into a tube containing 10 UPS units of heparin/ml blood and used either immediately or after storage (<48 hr, 4°C). Prior to each experiment, the blood was washed three times in PBS (150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4). Between the first and second washes, the cells were incubated for 30 min at 37°C to deplete intracellular glucose. After the third wash, the cells were diluted with PBS 150–200 times and used in the stopped-flow apparatus as described below. All of the sugar binding experiments were carried out in the zero-*trans* mode. Unless otherwise specified, the osmolality of all solutions was maintained at 300 mOsm. Osmolality was measured by a Fiske Osmometer (Model OS, Uxbridge, MA).

One-step (pink) ghosts were made by lyzing the washed red cells 1 : 20 in the osmotic lysis solution (5 mM Na_2HPO_4, pH 8.0, 0°C). After allowing hemolysis to proceed for 5 min on ice, the ghosts were centrifuged at 15,000 × *g* for 10 min and the red pellet was resuspended at 1 : 5 (vol/vol) in PBS, pH 7.4, and incubated at 37°C for 30 min to reseal. Two-step (white) ghosts were made from one-step ghosts by one additional wash of the red pellet with the lysis solution, before the resealing process. Both one- and two-step ghosts were prepared from the same red cells in order to determine the effect of this procedure on D-glucose binding.

To study the effect of ATP depletion on D-glucose binding, red cells (1 : 150 dilution) were initially depleted of glucose (30 min at 37°C) followed by an additional 50-min incubation at 37°C with either 2 mM ATP buffer (2 mM ATP, 10 mM Na_2HPO_4, pH 7.4 and sufficient NaCl to make a 300 mOsm solution) or PBS + 5 mM inosine and 5 mM iodoacetamide (ATP-depleted red cells). This was followed by an additional two washes in 2 mM ATP buffer or PBS buffer, before measurements of D-glucose binding kinetics (final concentration, 5 mM).

Cyt B inhibition of D-glucose binding was measured by incubating the red cells (dilution 1 : 175 with PBS) with freshly prepared 1 μM Cyt E and 0.2 or 2.0 μM Cyt B at 37°C for 20 min. The final ethanol concentration in the red cell solution was 0.05% and the same amount of ethanol was added to the control.

To study D-maltose inhibition of D-glucose binding, the washed red cell pellet was diluted with 10 mM (or 100 mM) D-maltose buffer (all subsequent sugar buffers consist of 10 mM Na_2HPO_4, pH 7.4, the specified sugar concentration, and NaCl sufficient to keep the total osmolality at 300 mOsm). After 10 min at room temperature, α-D-glucose binding kinetics were measured at 25°C with various D-glucose concentrations. For the dose-response curves of D-maltose, α- and β-D-glucose and α-D-galactose, the sugar concentration was varied while the osmotic pressure was maintained at 300 mOsm. Anomers of D-glucose were always prepared from the pure crystalline state (pyranose form only) and kept on ice in solution for not more than 7 hr. Based on the data of Carruthers and Melchior (1985), the half-time of mutarotation of α- and β-D-glucose at pH 7.4 is 308 ± 24 min and 401 ± 103 min, respectively, at 0.6°C. When we compared the binding kinetics of α-D-glucose (at 25°C) of freshly prepared solutions and solutions kept at 0°C until use (<7 hr), we found no difference. When this experiment was repeated, comparing freshly prepared solutions to solutions kept at 25°C for 7 hr, there were significant changes in the binding constants. For the freshly prepared α-D-glucose, $k_1 = 51 \pm 5.1$ sec^{-1}, $k_2 = 2.6 \pm 0.3$ sec^{-1}, and $k_3 = 2.4 \pm 0.3$ sec^{-1}. For the solution kept at room temperature for 7 hr, $k_1 = 59 \pm 1.3$ sec^{-1}, $k_2 = 4.9 \pm 0.9$ sec^{-1}, and $k_3 = 2.3 \pm 0.7$ sec^{-1}. In addition, the amplitude for this solution (room temp, 7 hr) was increased by 10% and the zero-time displacement was increased by 50%. Based on the data of Pigman (1957), the solution would have reached the equilibrium ratio of 36% α-anomer and 64% β-anomer after incubation at room temperature for 7 hr. Comparison of the results of both experiments leads us to the conclusion that measurement of the binding kinetics of sugar anomers by intrinsic trp fluorescence of GLUT1 is a true reflection of the state of mutarotation of the anomers. The two D-glucose anomer solutions were prepared freshly for each series of measurements. We assumed that the lifetime of α-D-glucose solutions was not more than 7 hr at 0°C (which would have led to 33% mutarotation) and never used the α-D-glucose solutions after that.

Fluorescence Measurements

Sugar binding was measured by intrinsic trp fluorescence measurements performed with a single beam stopped-flow spectrofluorimeter (Applied Photophysics model SF.17MV, Salisbury, England; [290 ± 3 nm, excitation; 340 ± 10 nm, emission (band-pass filter); observation cell volume: 100 μl]). The dead-time, which is governed by the software of the data acquisition system and the design of the solute mixing chamber, is of the order of 1.5 msec. All of the binding kinetics data were collected with 400 data points; 48–60 runs were averaged because the sugar binding signals never exceeded 5–10 mV. For studies of the temperature dependence of reactions, including α- and β-D-glucose binding to red cells, the sample compartment of the spectrofluorimeter was maintained at the desired temperature by use of a Constant Temperature Circulator (VWR Model 1145, Preston Industries, Niles, IL) which is accurate to ±0.1°C. We allowed 10–15 minutes for the system to equilibrate to the desired temperatures and equilibrated the loaded syringes with the waterbath for 5 min.

Control experiments previously reported by Janoshazi, Kifor and Solomon (1991) showed that changes in cell volume, induced by changed external osmolality, did not affect trp fluorescence. In one set of experiments, a range of osmolalities (after mixing) of 223 to 349 mOsm was found to produce no effect on trp fluorescence over a 200-sec period. Another set of experiments showed that volume changes induced by glucose transport did not produce secondary light-scattering effects, which were measured at 430 nm.

The Stern-Volmer quenching plots of GLUT1 intrinsic trp fluorescence by D-glucose were assumed to be linear over the D-glucose concentration range used in our experiments. A fitting routine was used to fit the data for D-glucose and D-galactose binding by nonlinear least squares to three exponentials plus a constant $[a_1e^{(-k_1t)} + a_2e^{(-k_2t)} + a_3e^{(-k_3t)} + a_4]$. The initial displacement of the trp fluorescence amplitude, when t is essentially 0, is given by $\Sigma_i a_i$. The amplitude and time constant of the fast expo-

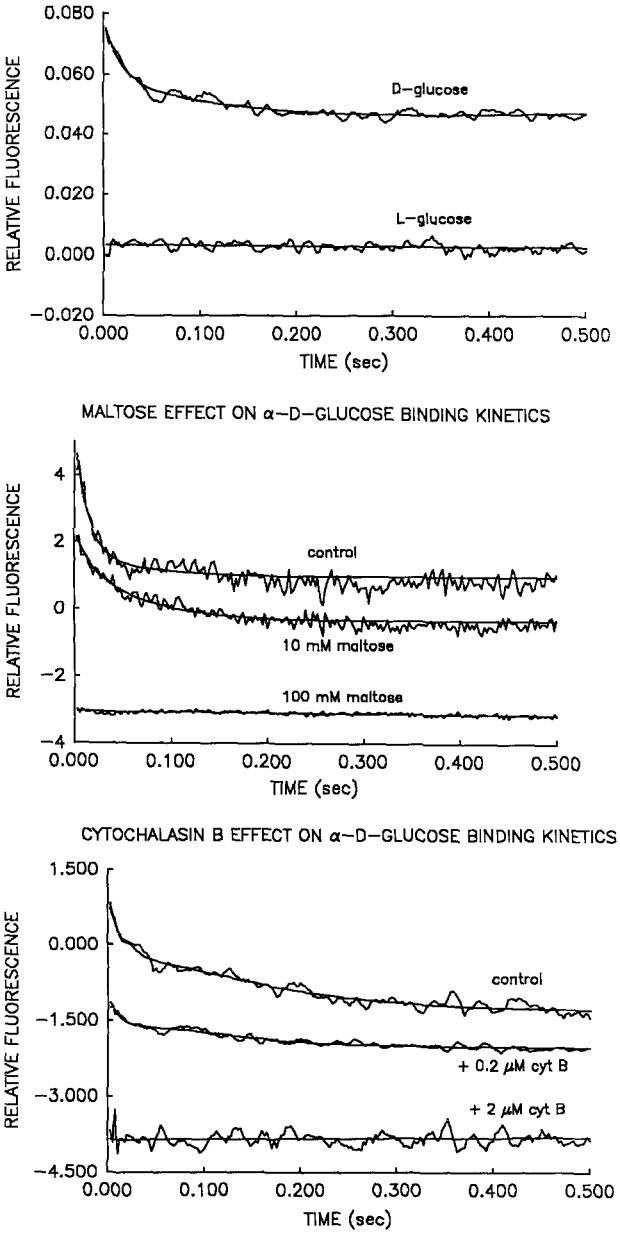

Fig. 1. (Top) Binding kinetics of D- and L-glucose to red cells at 24°C. Red cells (1 : 150 dilution) were mixed with either D- or L-glucose (final concentration, 7.5 mM; baseline subtracted; D-glucose curve shifted down 2.0 units; shifts are carried out by changing the adjustable constant in the equation for the fitted curve so that, when a shift is described by its position on the y axis, the value given is the equilibrium value.) Rate constants: $k_1 = 60.0 \pm 0.8$ sec^{-1}; $k_2 = 2.2 \pm 0.7$ sec^{-1}; $k_3 = 5.8 \pm 1$ sec^{-1}. One of five experiments. (Middle) D-maltose inhibition of 7 mM D-glucose binding kinetics at 24°C. Partial inhibition by 10 mM D-maltose; complete inhibition by 100 mM D-maltose. Control shifted from 6.12; 10 mM D-maltose shifted from −0.025; 100 mM D-maltose shifted from 0.122. One of three experiments. (Bottom) Effect of cytochalasin B on α-D-glucose binding kinetics. Washed red cells (1 : 175 dilution) were incubated with freshly prepared 1 μM Cyt E and either 0.2 or 2.0 μM Cyt B for 20 min at 37°C (control cells were incubated with 0.05% v/v ethanol). Kinetics were measured at 20°C; each curve is the average of 48 runs. The control and the highly quenched 2 μM Cyt B treated curves have been shifted for better visibility (control shifted from 6.2 units; 2 μM Cyt B curve shifted from −7.8 units). One of two experiments. Rate constants (sec^{-1}): **Control:** $k_1 = 78 \pm 2$; $k_2 = 19 \pm 1$; $k_3 = 9 \pm 0.2$. **Control + 0.2 μM Cyt B:** $k_1 = 57 \pm 2$; $k_2 = 29 \pm 1$; $k_3 = 13 \pm 1$.

nential were obtained by fitting the first ≈100 msec to a single exponential. With these two parameters set, the initial condition for the second exponential were: negative amplitude; second exponential rate constant very much smaller than the first. With these conditions a fit could be obtained over the first 500–700 msec. Having now set the first four parameters, the entire 1-sec time course was fit to three exponentials plus a constant and the remaining three parameters were obtained. This procedure gave a first-order approximation of all seven constants, whose values were then used as the initial estimates for a seven-parameter fit, which provided the final values used in the tables. When there was no difference between the second and third exponentials, as for 4,6-O-ethylidene-D-glucose and D-maltose, the data were fit to two exponentials plus a constant. The validity of our method of data reduction is attested by the fit of the data, as exemplified in Fig. 3 in which each k_i fits its own theoretical curve over two decades of concentration. Since the rate constants are idiosyncratic to the individual blood and experimental condition, there is an individual control for each experiment.

Results and Discussion

Control Experiments to Test the Validity of the Tryptophan Fluorescence Enhancement Method

The solutes whose binding elicits the trp fluorescence changes we observe are sugars and sugar analogues which are known to bind to GLUT1, so it is logical to assign the resultant changes in trp fluorescence to GLUT1. However, there are trp residues in other membrane proteins in addition to the six trp residues in GLUT1 (Mueckler et al., 1985) as, for example, the 12 trp residues in band 3 (Lux et al., 1989). Therefore, it is necessary to make sure that the trp residues in GLUT1 are the ones responsible for the fluorescence time course we observe. To check the validity of this attribution, we have carried out a number of control experiments. The comparison of 7.5 mM D- and L-glucose binding kinetics in Fig. 1 (top) shows that the kinetic component of trp fluorescence enhancement caused by D-glucose is absent with L-glucose, in agreement with Carruthers (1986) finding that L-glucose does not bind to stripped ghosts.

Although D-maltose is a nontransportable sugar, it binds to GLUT1 on the extracellular face of the cell with $K_d = 2.6 \pm 0.1$ mM and competes with D-glucose binding (Lacko & Burger, 1962; Carruthers, 1986). Figure 1 (middle) shows that the ini-

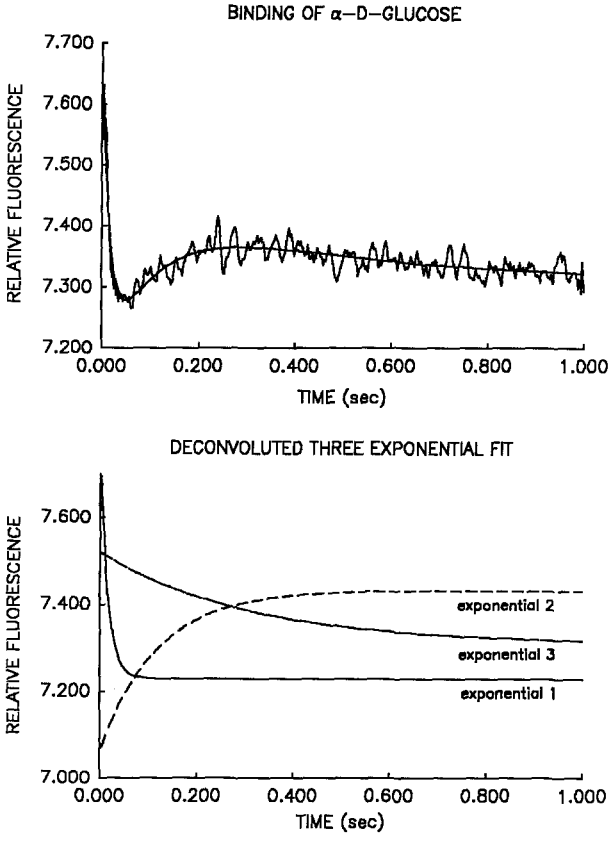

Fig. 2. (Top) Binding kinetics for α-D-glucose at 25°C. Red cells (1 : 150 dilution) were mixed with freshly prepared α-D-glucose under zero-trans conditions (final concentration 4 mM, average of 44 runs, baseline subtracted). The kinetic parameters for the fitted curve have been derived from the three-exponential fit shown below. (Bottom) Deconvoluted, three-exponential fit for data in Fig. 2 (Top). The rate constants are (sec^{-1}): $k_1 = 62 \pm 6$; $k_2 = 8.4 \pm 3.7$; and $k_3 = 3 \pm 2$.

tial rate of 7.0 mM D-glucose binding is reduced when 10 mM D-maltose is added and is entirely suppressed by 100 mM D-maltose.

The mold metabolite, cytochalasin B (Cyt B), binds to an intracellular site on GLUT1 to inhibit D-glucose uptake specifically (Lowe & Walmsley, 1989). We have found that high (2 μM) Cyt B concentrations completely suppress D-glucose-induced fluorescence kinetics, as shown in Fig. 1 (bottom). We used a lower Cyt B concentration (0.2 μM) to inhibit D-glucose uptake partially so that we could measure the inhibition kinetics (zero-*trans* conditions). We found, as will be discussed below, that Cyt B noncompetitively inhibits the effects of D-glucose binding on k_1, consistent with the noncompetitive inhibition observed under zero-*trans* influx conditions by Lowe and Walmsley (1989).

The specificity of GLUT1 resides in its ability to recognize the conformation of the bound sugar and to discriminate among enantiomers. In these critical aspects of GLUT1 specificity, the results obtained from the kinetics of trp fluorescence enhancement mirror results obtained by more conventional assays. Trp fluorescence-quenching kinetics distinguishes between glucose enantiomers (Fig. 1, top); shows that D-maltose can inhibit D-glucose binding (Fig. 1, middle); and can recognize the conformation of the specific glucose transport inhibitor, Cyt B (Fig. 1, bottom). These three control experiments show that analysis of trp fluorescence kinetics provides a satisfactory probe of GLUT1, in that the results reflect the GLUT1 conformational changes which accompany the binding of D-glucose.

Characterization of D-Glucose Binding

Although many investigators as, for example, Krupka and Devés (1981), Widdas (1988), and Lowe and Walmsley (1989) conclude that red cell glucose transport can be accounted for by a carrier model in which the glucose transport site alternates between the two faces of the membrane (alternating conformer model), others, notably Carruthers (1990), believe that glucose transport can only be accounted for by the fixed site model in which sites on both faces of the membrane can be occupied by ligand simultaneously. Our experiments were not designed to attempt a resolution between these conflicting views.

The process of D-glucose binding and transport begins with a virtually instantaneous displacement of the trp fluorescence amplitude; this is followed by a set of reaction steps over the first second whose resolution depends upon rapid reaction methods. Fig. 2 (top) shows the time course of the trp fluorescence following binding of 4 mM α-D-glucose at 25°C. The zero-time point is at a relative fluorescence of 7.64, which represents a zero-time enhancement from the baseline (relative fluorescence = 0), too fast for us to measure. We were unable to resolve this component even after lowering the temperature to 5°C, which shows that its time constant is probably less than 3 msec at this temperature. To avoid confusion with the exponential kinetics of the subsequent events, we denote the zero-time component as a "displacement". In Fig. 2 (top) the zero-time displacement is much larger than the subsequent excursions which reduce the relative fluorescence by 0.32 units to 7.32 units (fitted curve, 1 sec); this disparity between the amplitude of the zero-time displacement and the subsequent exponential kinetics is consistently observed at 20–25°C. The sign and magnitude of the zero-

time displacement are very temperature dependent, and idiosyncratic to the binding sugar. For example, at 25°C, where α-D-glucose and D-maltose exhibit a positive (enhancing) displacement, 4,6-O-ethylidene-D-glucose quenches.

The existence of the three exponential components in the early stages of D-glucose binding to fresh red cells can be inferred from the convoluted nature of the time course. The three processes have been deconvoluted in Fig. 2 (bottom), which shows that the early fast quenching arises from a component with a rate constant, $k_1 = 62 \pm 6$ sec^{-1}. This reaction comes to equilibrium within the first 300 msec and makes no further contribution to the kinetics. The second and third exponentials are slower and the deflections are in opposite directions. The second process is a fluorescent enhancement with $k_2 = 8.4 \pm 3.7$ sec^{-1} and the third process is a quenching component with $k_3 = 3 \pm 2$ sec^{-1}. The relative contributions of the three separate exponentials are dependent upon the characteristics of the specific blood, particularly with respect to the amplitude of exponential 2 which is very sensitive to temperature. Thus, though our D-glucose and D-galactose binding data are always fit to the three exponentials, the curves at temperatures below 22°C often look, at first sight, almost as if they consisted of two exponentials.

There are only a small number of rapid reaction measurements in the literature which might have permitted direct comparison with our data. Appleman and Lienhard (1985) applied the stopped-flow method to study the time course of trp fluorescence enhancement subsequent to 4,6-O-ethylidene-D-glucose binding to purified red cell glucose transporter. The fluorescence quenching they observed could be described with a single exponential with $k_{obs} \approx 100$ sec^{-1} for 25 mM 4,6-O-ethylidene-D-glucose binding at 25°C. Proton NMR was used by Wang et al. (1986) to study β-D-glucose binding to leaky red cell ghosts at 23°C, but the 1-sec resolution of the NMR method was not fast enough to reveal the details of the initial binding steps, though Wang et al. calculate that the binding rate constant is about 10^3 sec^{-1}. Lowe and Walmsley (1986) studied D-glucose binding kinetics by a rapid reaction method which gave data covering the first 10–100 msec. They did not fit their data with individual exponentials, as we have done. Instead, they obtained their kinetic parameters by fitting their data to a version of the alternating conformer model which had been simplified by the assumption that the initial binding steps were too fast to measure. Thus, no direct quantitative comparison is possible with our exponential rate constants.

The time constant of the zero-time displacement may be presumed to be <1 msec at 25°C so that the bimolecular association of ligand and membrane, which it presumably represents, could be equated with Wang et al.'s binding step, whose rate constant is calculated as 10^3 sec^{-1}.

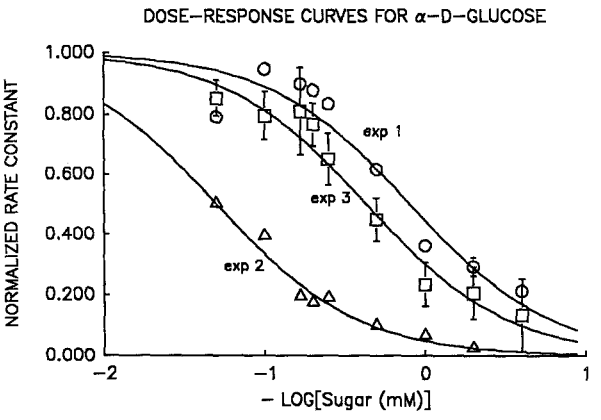

Fig. 3. Binding kinetics for α-D-glucose to red cells at 20°C. Red cells (1 : 150 dilution) were mixed with varying concentrations of freshly prepared α-D-glucose and the kinetics determined. Binding kinetics at each sugar concentration were determined by the average of 44–60 runs, baseline subtracted. The normalized rate constants derived from each of the three exponentials in the three-exponential fit were plotted separately as a function of −log[sugar]. For the first exponential (○), $K_d = 1.1 \pm 0.7$ mM; for the second exponential (△), $K_d = 18.6 \pm 8.2$ mM; for the third exponential (□), $K_d = 1.9 \pm 0.7$ mM. When error bars are not shown, they are within the points. One of three experiments.

We have carried out a series of dose-response experiments to characterize the three exponentials for α-D-glucose binding and obtained the results shown in Fig. 3. The validity of the deconvolution process used to measure the kinetics of these three exponential reactions is supported by the reproducible nature of the data in this figure, particularly the observation that each of the k_i's fits its own theoretical dose-response curve over a wide range of concentrations. The K_d for exponential 1 in this series of experiments is 1.1 ± 0.7 mM, close to the K_d for exponential 3, 1.9 ± 0.7 mM, a similarity which is broadly applicable to the transported sugars. The K_d for binding of the second exponential component is an order of magnitude less tight, 18.6 ± 8.2 mM. These dissociation constants may be related to the two D-glucose binding sites that Carruthers (1986) reported in his study of the purified transporter, with K_d's = 1.5 ± 0.2 mM and 26.9 ± 3.2 mM.

Another series of experiments on a different blood were carried out to compare the epimers, D-glucose and D-galactose. The K_d for D-glucose binding to fresh red cells (zero-*trans* conditions) was determined from the concentration dependence of the normalized rate constant of the first exponen-

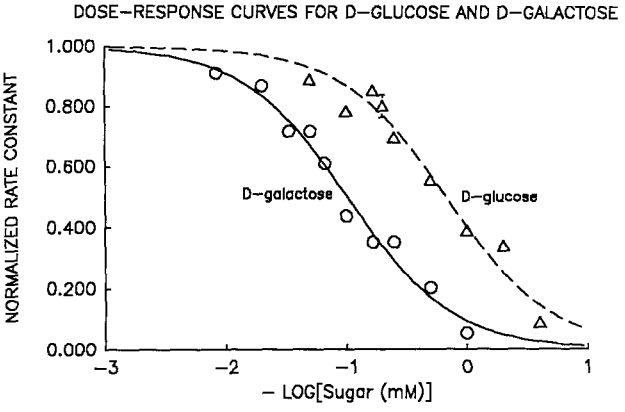

Fig. 4. Dose-response curve for binding of α-D-glucose (△) and α-D-galactose (○) to red cells at 20°C. The error bars are contained within the points. The normalized rate constants were derived from the first exponential in the three-exponential fit. $K_{d,glu}$ = 1.5 ± 0.5 mM; $K_{d,galac}$ = 9.7 ± 2.4 mM. One of two experiments.

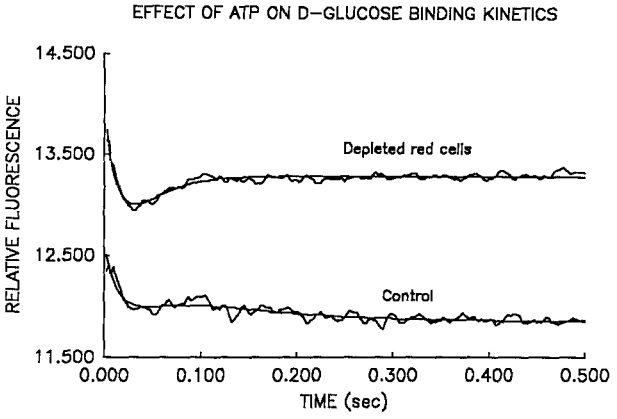

Fig. 5. Effect of ATP depletion on the binding kinetics of D-glucose (2.5 mM) at 19.8°C. Red cells (1 : 150 dilution) were initially depleted of glucose followed by an additional 50 min incubation at 37°C with either 2 mM ATP buffer (control) or PBS + 5 mM inosine and 5 mM iodoacetamide (depleted cells). The position of the control curve on the y axis has been shifted from eight units for better visibility. Rate constants (sec^{-1}): **Depleted:** k_1 = 66 ± 3; k_2 = 32 ± 2; k_3 = 27 ± 10. **Control:** k_1 = 55 ± 4; k_2 = 30 ± 3; k_3 = 9.8 ± 0.2.

tial, k_1, and found to be 1.5 ± 0.5 mM, as shown in Fig. 4, in good agreement with Carruthers' (1986) figure of 1.5 ± 0.2 mM and Lowe and Walmsley's (1986) value of 1.6 ± 0.4 mM, obtained by tracer methods. The epimer, D-galactose differs from D-glucose by the orientation of a single -OH group at C-4, which is nonetheless sufficient to change its K_d to 9.7 ± 2.4 mM, as shown in Fig. 4. This figure is in good agreement with Carruthers' (1986) value of 7.4 ± 0.5 mM (steady-state fluorescence enhancement) and Ginsburg and Stein's (1975) value of 11.2 ± 6.4 mM (tracer methods). Our finding that the difference between the epimers at C-4 is reflected in the binding kinetics is consistent with the view of Barnett et al. (1973) and Kahlenberg and Dolansky (1972) that H-bonding to the D-glucose C-4 stabilizes the D-glucose/transporter complex. This ability to discriminate between epimers provides another illustration of the validity of the fluorescence enhancement method.

Although the uptake of D-glucose is characterized as facilitated diffusion rather than active transport because D-glucose is not accumulated against a concentration gradient, the uptake is not independent of metabolic energy. In the red cell, ATP has an additional role beyond the provision of metabolic energy to drive active transport processes: the phosphorylation of intracellular and membrane proteins as, for example, band 3 (Low et al., 1987). We have depleted red cells of ATP by the method of Lew (1971) to study the effect of ATP depletion on the initial kinetics of D-glucose binding. From the time course shown in Fig. 5 and the rate constants given in the figure legend, it can be seen that energy depletion affects the first and third exponentials, but k_2 does not change. The effect on the first exponential which is clearly visible in Fig. 5 is primarily due to the increased amplitude of the first term, which is 40% greater in the depleted cells, while there is little or no effect on the rate constant, k_1 (*see* legend). ATP depletion causes a very large change in the third exponential, increasing k_3 from 9.8 ± 0.2 sec^{-1} in untreated controls, to the very much faster rate constant of 27 ± 10 sec^{-1} in the depleted cells, consistent with the sevenfold increase in V_{max} for sugar uptake, induced by ATP depletion (Carruthers, 1990).

The effect of ATP on the first exponential is controlled by events at the extracellular face, as will be shown in the following sections. Yet the ATP site itself is on or near the cytosolic face of the membrane, as shown by Carruthers' (1986) finding of nucleotide binding sites in the sugar transport protein through which ATP modifies the catalytic properties of the protein. Thus, the ATP depletion experiments provide another example of information transfer across the membrane, in which ATP binding on the cytosolic face of GLUT1 leads to conformational changes of the sugar binding site of the extracellular face, similar to the effect on extracellular D-maltose binding exercised by intracellular Cyt B (Helgerson & Carruthers, 1987).

Importance of Native Red Cell Conformation

Drastic treatments of the red cell, such as those involved in making ghosts, lead to substantial

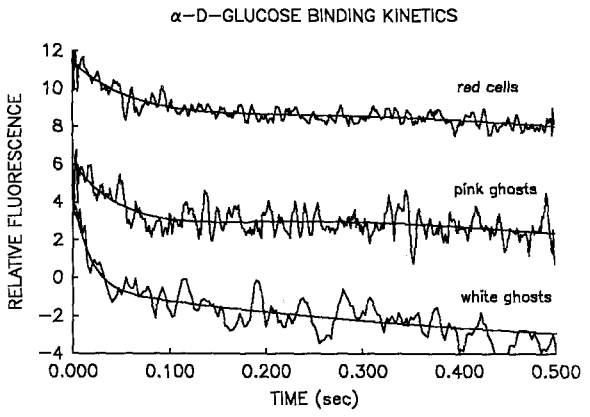

Fig. 6. Comparison of the kinetics of 1 mM α-D-glucose binding to intact red cells, one-step (pink), and two-step (white) ghosts at 20°C. Pink and white ghosts were prepared from the red cells used for the top curve. Taking the baseline fluorescence (cells + PBS) as a reference, ghost suspensions were diluted appropriately to achieve the same constant trp fluorescence before mixing with sugar buffer. Each curve is the average of 10–50 runs, baseline subtracted (signal amplitudes for the red cells and pink ghosts have been multiplied by factors of 5 and 2, respectively, and the curves have been shifted on the y axis for better visibility). One of three experiments. Rate constants (sec^{-1}): **Red cells:** $k_1 = 10.3 \pm 1.2$; $k_2 = 5.2 \pm 0.6$; $k_3 = 3.1 \pm 0.6$. **Pink ghosts:** $k_1 = 12.9 \pm 2.2$; $k_2 = 7.8 \pm 2.2$; $k_3 = 2.8 \pm 1.8$. **White ghosts:** $k_1 = 56 \pm 2$; $k_2 = 50 \pm 3$; $k_3 = 2.1 \pm 0.4$.

changes in the time constants of the binding exponentials, in agreement with observations made previously by steady-state methods (Janoshazi et al., 1991). The stopped-flow time courses of α-D-glucose binding over the first 0.5 sec at 20°C are shown in Fig. 6, and the rate constants are given in the figure legend. As we proceed from the native cell through the one-step ghosts and end with white ghosts, the number of washes increases and the treatment becomes increasingly severe. Qualitative examination of Fig. 6 shows that, as the red cell structure becomes increasingly perturbed, the initial steps of ligand binding are faster and their relative amplitude is greater. Nonetheless, as reported by Challiss, Taylor and Holman (1980) the V_{max} of D-glucose influx (zero-*trans*) is relatively unaffected, going from 30.8 ± 2.2 mM min^{-1} with red cells to 25.5 ± 3.2 mM min^{-1} with pink ghosts.

There are large changes in the first exponential, whose time constant increases by a factor of five between red cell and white ghosts and even larger in the second exponential, where there is an order of magnitude increase. As the number of washes used in the preparation increases, the rate constant for reaction 1 increases. Since this is the first reaction in the sequence, its increased rate would be consistent with increased access of D-glucose to the binding site, perhaps as a result of removal of a protective element, possibly a lipid. Such behavior would be consistent with Pawagi and Deber's (1990) model in which the GLUT1 trp fluorescence characteristics depend on one or more trp residues which can be located alternatively in an aqueous or membrane domain. When the glucose transport protein is separated and purified, its new environment is vastly different from the native state. This changed environment presumably accounts for the difference between the fluorescence kinetics of 4,6-O-ethylidene-D-glucose binding to the separated transporter observed by Appleman and Lienhard (1985) and for our results in the native red cell, discussed below. These considerations mean that great care must be exercised when results obtained in experiments in reconstituted preparations are taken to be representative of the behavior of the native transporter in its normal environment.

Discrimination between the α- and β-D-Glucose Anomers

Mutarotation of the α- and β-D-glucose anomers introduces practical difficulties in measurements of binding and transport of the individual anomers, because the half-time for mutarotation at pH 7.4 ranges from 10 min at 37°C to 400 min at 0°C (Carruthers & Melchior, 1985). This time course is approximately the same as the glucose flux in the temperature range of 0–40°C, so conventional flux measurements may not be suitable for experiments with nearly pure α- and β-anomers, unless special precautions are taken. Our direct method of deconvoluting trp kinetics induced by D-glucose binding makes it possible to distinguish between the anomers, as can be seen in Fig. 7 (top). The two individual kinetic responses of exponential 1 at 9.3°C have been completed before significant interconversion occurs. The difference between our results and those of Carruthers and Melchior (1985), who found no difference between the initial phase of α- and β-D-glucose uptake at 0.66°C, measured over a time span in which mutarotation was negligible, may be ascribed to the fact that they measured uptake while we measured the initial phases of ligand binding. Most of the other studies in the literature are flux measurements, but the data are conflicting, some authors reporting a difference between anomer fluxes and others finding none (Faust, 1960; Fujii et al., 1986; Miwa et al., 1988). Different problems arise when D-glucose flux is measured by an indirect method as in Appleman and Lienhard's (1989) study based on the kinetics of 4,6-O-ethylidene-D-glucose binding to the purified transporter.

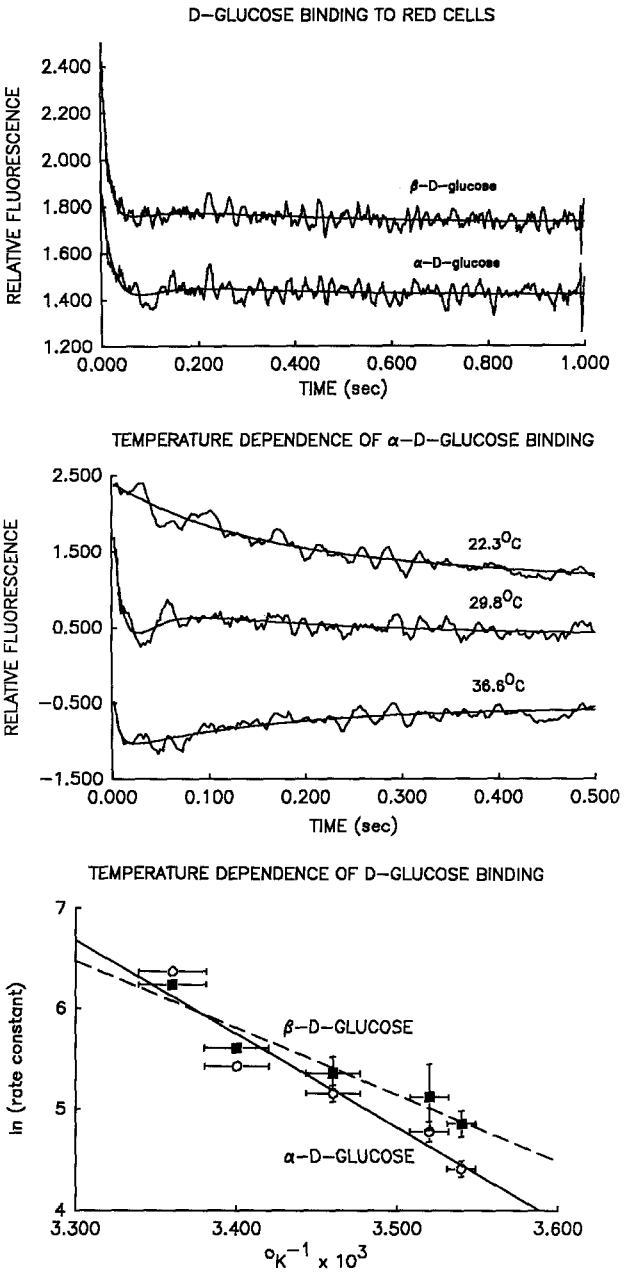

Fig. 7. (Top) Binding kinetics of α- and β-D-glucose to intact red cells. Red cells (1 : 150 dilution) were mixed with freshly prepared 5 mM α- or β-D-glucose at 9.3°C and the binding kinetics were determined. Each curve is the average of 48 runs; the baseline (red cells in PBS, zero relative fluorescence) has been subtracted. One of three experiments. Rate constants (sec^{-1}): **α-D-glucose:** $k_1 = 34 \pm 5$; $k_2 = 17 \pm 6$; $k_3 = 4 \pm 3$. **β-D-glucose:** $k_1 = 75 \pm 7$; $k_2 = 8 \pm 4$; $k_3 = 5 \pm 1$. (Middle) Binding kinetics of α-D-glucose to intact red cells as a function of temperature. Red cells (1 : 150 dilution) were mixed with freshly prepared α-D-glucose (final concentration 7.5 mM). Each curve (baseline subtracted) is the average of 50–100 runs, and baselines were repeated after every 50 runs at each specific temperature. One of three experiments. Rate constants (sec^{-1}): **22.3°C:** $k_1 = 12 \pm 3$; $k_2 = 13 \pm 4$; $k_3 = 1.5 \pm 0.4$. **29.8°C:** $k_1 = 81 \pm 8$; $k_2 = 54 \pm 6$; $k_3 = 4.5 \pm 0.8$. **36.6°C:** $k_1 = 147 \pm 6$; $k_2 = 147 \pm 6$; $k_3 = 6 \pm 0.5$. (Bottom) Arrhenius plots for the binding of α- and β-D-glucose to red cells. Binding kinetics for freshly prepared α- and β-D-glucose (5 mM final concentration) were determined as a function of temperature. Average of two experiments. Rate constants derived from the first exponential of the three-exponential fit were used for the Arrhenius plot. The activation energy for α-D-glucose (○) is 18.4 ± 0.8 kcal mol^{-1} and for β-D-glucose (■) is 13.6 ± 1.4 kcal mol^{-1}. (Correlation coefficients are 0.91 and 0.89 for the α and β-D-glucose data, respectively). The lines cross at ≈23.5°C.

The fact that the exponential 1 rate constants for the two anomers differ by about a factor of two (*see* legend) suggests that this reaction is sensitive to the formation of an H-bond between GLUT1 and the anomeric -OH group. For the second reaction, the rate constant of the α-anomer (at 9.3 °C), 17 ± 6 sec^{-1}, is apparently greater than that of the β-anomer, 8 ± 4 sec^{-1}. This selectivity suggests that the formation of the H-bond to the anomer -OH modifies the environment of the trp residues probed by exponential 2. There is no difference in the rate constants of the third reaction.

The temperature dependence of α-D-glucose binding kinetics shown in Fig. 7 (middle) illustrates the interdependence among the exponentials, whose rate constants are given in the legend. The rate constants of all three exponentials rise smoothly with temperature but the rate constant of the third exponential remains at least one order of magnitude slower than the others. The interplay between the exponentials and the dominance of exponential 2 is illustrated by the fact that, at 36.6°C, the curve rises over the period between 0.1 to 0.5 sec, showing that fluorescence is enhanced, while at 22.3°C, where exponentials 1 and 3 contribute significantly to this phase of the reaction sequence, fluorescence is quenched.

The temperature dependence of the individual exponentials given in the legend to Fig. 7 (middle) may be compared with that determined by Lowe and Walmsley (1986) for the V_{max} of zero-*trans* D-glucose influx computed from initial fluxes in a rapid reaction apparatus. Their observation that the data do not fall on a linear Arrhenius plot shows that more than a single rate determining step is involved, consistent with our conclusions. For comparative purposes, a qualitative measure of the temperature dependence of V_{max} can be obtained from the ratio of $V_{max,36.6°C}$ to $V_{max,22.3°C}$, of 12.3, as estimated from Fig. 2 in Lowe and Walmsley (1986). Our comparable ratios, computed from the Fig. 7 (middle) data for α-D-glucose binding are: k_1, 12.3; k_2, 11.3; and k_3, 4. The general similarity of these ratios is consistent with the view that the Lowe and Walmsley V_{max} contains contributions from our three exponential processes.

The differences between the α- and β-anomers are also very sensitive to temperature, even though the β-D-glucose zero-time displacement is always greater than that of the α-anomer, independent of temperature. We have measured the activation energies of exponential 1 (Fig. 7, bottom) and have found not only that the activation energy for the α-anomer, 18.4 ± 0.8 kcal mol^{-1}, is greater than that of 13.6 ± 1.4 kcal mol^{-1} for the β-anomer, but that the lines cross. This difference in activation energy means that it is significantly easier for the β-anomer to bind to the transporter. In their 1973 study of the structural requirements for sugar binding, Barnett et al. used fluorinated analogues that cannot mutarotate to measure the binding and provided evidence that H-bonds were preferentially formed to the -OH group in the β conformation. It is gratifying that our kinetic measurements provide support for their equilibrium determinations.

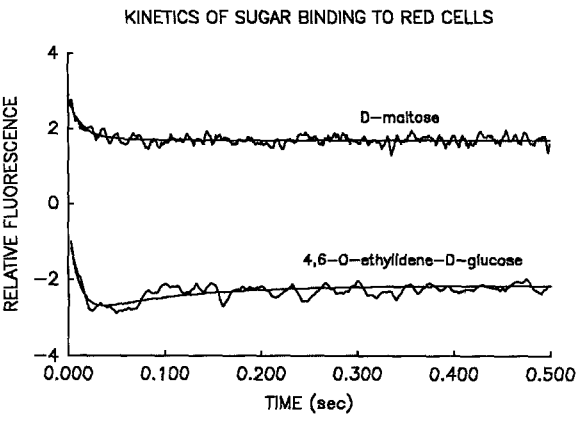

Fig. 8. Binding kinetics for D-maltose and 4,6-O-ethylidene-D-glucose at 24°C. Red cells (1 : 160 dilution) were mixed with either D-maltose (45 runs) or 4,6-O-ethylidene-D-glucose (10 runs) (20 mM final concentration, baseline subtracted). The maltose data have been scaled by a factor of ten for better visibility. One of two experiments. Rate constants (sec^{-1}): **D-maltose:** k_1 = 102 ± 24; k_2 = 47 ± 13; **4,6-O-ethylidene-D-glucose:** k_1 = 101 ± 14; k_2 = 10 ± 1.

Relation of Exponential Time Constants to Binding and Transport Events

In order to assign possible roles to the three exponential processes whose time constants we have determined, we constructed a rough, working scaffold with which to envision these roles. Since there are six trp residues in GLUT1 and ligand binding sites at both faces of the membrane, there is no way to assign any particular trp residue, or group of residues, to any specific exponential process. We are unable even to determine whether the reactions are sequential or parallel, so that there are no effective restraints to guide us in trying to construct a reaction scheme, much less to make a confident assignment of any reaction to a specific step in either the alternating conformer or the fixed site model. Instead, we have given each reaction a name which describes its putative role in the transport process and will try to justify each name by relating it to our experimental findings.

Exponential 1 is the "hello" reaction; exponential 2 is the "recognition" reaction; and exponential 3 is the "transport-related" reaction. The hello is the greeting that GLUT1 offers, after the binding displacement, to the broad class of eligible sugars, independent of their conformation or even of the number of saccharides which compose them. But hello does not extend its greetings to sugars of the wrong enantiomeric configuration, like L-glucose, which does not bind to GLUT1. The hello rate constant ranges between about 13 to 70 sec^{-1} at 20°C (sugar concentration about 5 mM) and the range is smaller when comparisons are restricted to blood from a single individual. The recognition reaction, exponential 2, discriminates critically among the sugars and is responsible for selecting those which will be transported, and rejecting the rest. Exponential 2, usually associated with fluorescence enhancement, has the most variable rate constant, ranging over more than an order of magnitude from about 1 to 40 sec^{-1} at 20°C. Sugars which fail the recognition test do not elicit more than two exponential steps. The transport-related exponential 3 is the slowest reaction, with a rate constant in the range of 0.5 to 10 sec^{-1}.

Although we have given distinctive names to the three exponentials for discussion purposes, it must be remembered that all the six trp residues are in the same GLUT1 molecule. In principle, it is unlikely that a change in the environment of one trp residue will not be communicated to other regions in the transporter. Thus, the hello and recognition processes should be considered as two linked aspects of a complex identification procedure.

Studies of the Nontransportable Sugars and Cyt B

4,6-O-ethylidene-D-Glucose: Binding of this nontransportable sugar to whole red cells at 24°C is characterized by two exponential kinetics, as shown in Fig. 8; the fast rate constant (*see* legend) for a quenching component of 101 ± 14 sec^{-1} is assigned to the hello reaction, and the slower rate constant of an enhancing component, 10 ± 1 sec^{-1},

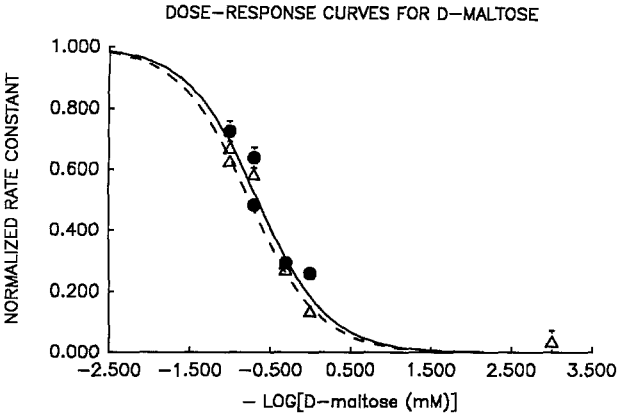

Fig. 9. Comparison of the dose-response curves of the first and second exponentials for D-maltose binding at 25°C. Red cells (1 : 150 dilution) were mixed with varying concentrations of D-maltose. The normalized rate constants for each of the exponentials in a double exponential fit were then plotted separately as a function of D-maltose concentration. The K_d derived from exponential 1 (△) is 4.9 ± 1.9 mM, and that from exponential 2 (●) is 3.9 ± 2.4 mM. One of three experiments.

Table 1. Effect of D-maltose and Cyt B on D-glucose binding kinetics (exponential 1)

	K_d mM	V_{max} sec^{-1} (relative units)
D-glucose	0.54 ± 0.11	58 ± 13*
+ 10 mM D-maltose	1.40 ± 0.02	70 ± 5
D-glucose	0.9 ± 0.7	106 ± 25
+ 0.2 μM Cyt B	1.0 ± 0.7	66 ± 15

* Difference not significant ($P < 0.2$, t-test)

is assigned to the recognition reaction. The absence of the third exponential term in this nontransportable sugar is consistent with our assignment of the third exponential to a transport-related process. Although Appleman and Lienhard (1985) found a rate constant of 100 sec^{-1} for 4,6-O-ethylidene-D-glucose binding, their rate constant was associated with a quenching reaction, whereas ours consist of a fast fluorescence quenching followed by a slower enhancing. This difference may be ascribed to the influence of the environment on the transporter, as discussed above.

D-Maltose: Binding (measured on the same blood and at the same 20 mM concentration and 24°C temperature) can also be described by two exponentials. The rate constant of the hello reaction, 102 ± 24 sec^{-1} is the same as that for 4,6-O-ethylidene-D-glucose and similar to that of D-glucose (72 ± 7 sec^{-1}) measured under the same conditions. Not only is the recognition reaction rate constant (47 ± 13 sec^{-1}) much faster than that for 4,6-O-ethylidene-D-glucose (10 ± 1 sec^{-1}), but also the fluorescence amplitude of exponential 2 for D-maltose is quenched rather than enhanced, as for 4,6-O-ethylidene-D-glucose. Clearly, the recognition reaction is very sensitive to ligand conformation.

The D-maltose dissociation constant for the hello reaction has K_d = 4.9 ± 1.9 mM, as shown in Fig. 9, in good agreement with Carruthers' (1986) value of 2.9 ± 0.1 mM in stripped ghosts (his low apparent K_d) and virtually the same as that for the recognition reaction, 3.9 ± 2.4 mM. 4,6-O-ethylidene-D-glucose appears to behave similarly, but there are not enough data to make a dose-response curve. This similarity may be contrasted with transportable sugars, in which the K_d's of the hello and transport-related reactions, shown in Fig. 3, are closely similar, while that of the recognition reaction differs by an order of magnitude.

Competition Experiments: The K_d and V_{max} for the effect of 10 mM D-maltose on the hello reaction for D-glucose binding at 20°C were measured and compared with the K_d and V_{max} for the effect of 0.2 μM Cyt B. D-maltose appeared to be a competitive inhibitor of D-glucose binding, since in one experiment (of two), as shown in the Table, K_d changed from 0.54 ± 0.11 mM to 1.40 ± 0.02 mM while V_{max} did not change significantly. When the D-maltose concentration is increased to 100 mM, D-glucose binding kinetics is completely suppressed. These observations of competitive binding support the view that the environment of the trp residues probed by exponential 1 is controlled by the D-glucose binding site on the outer membrane face.

To study the effect of 0.2 μM Cyt B on D-glucose binding (in the presence of Cyt E), we also measured the V_{max} and K_d of exponential 1. In this case, the Table shows the inhibition to be noncompetitive since K_d remains the same, while relative V_{max} changes [$P < 0.05$, t-test] from 106 ± 25 sec^{-1} to 66 ± 15 sec^{-1}. Since the known binding site for Cyt B is on the cytosolic face of the membrane, it can only affect the extracellular D-glucose binding site by an allosteric mechanism. Thus, the noncompetitive Cyt B inhibition is consistent with, and supportive of, our view that the environment of the trp residues probed by exponential 1 is controlled by the extracellular D-glucose binding site. No such definitive information is available for exponential 2 since the inhibition appears to be mixed for both D-maltose and Cyt B. The data for exponential 3 are too noisy to permit firm conclusions.

Generalities Concerning the "Time Constant/Transport Function" Relationship

When these experiments on nontransportable sugars are considered, together with those on D-glucose and with Cyt B, a number of generalities emerge.

(i) The hello reaction appears to be at or near the extracellular face. The two nontransportable sugars, 4,6-O-ethylidene-D-glucose and D-maltose must interact with GLUT1 on the outside membrane face; both elicit hello reactions. Furthermore, when D-maltose interacts with GLUT1 to inhibit D-glucose binding, the inhibition is competitive.

(ii) Since the rate constant of the D-glucose transport-related reaction (exponential 3) is changed dramatically by cytosolic ATP depletion, the reaction is controlled by an element in contact with the cytosolic surface.

(iii) The hello reaction is relatively insensitive to the conformation of the binding sugar. This generality arises from the equality of the time constants for the hello reaction for 4,6-O-ethylidene-D-glucose (101 ± 14 sec^{-1}) and D-maltose (102 ± 24 sec^{-1}), which are close to that for D-glucose, 72 ± 7 sec^{-1}. Yet when the changes are large enough, such as between the anomeric -OH groups of α-D-glucose and β-D-glucose, there are effects on the hello reaction kinetics.

(iv) The generality that the recognition rate constant is very sensitive to the sugar conformation is put forward because the recognition rate constant for D-maltose is 47 ± 13 sec^{-1}, five times faster than that for 4,6-O-ethylidene-D-glucose (10 ± 1 sec^{-1}). Similarly, there is a twofold difference in the recognition rate constants (exponential 2) between α-D-glucose and β-D-glucose.

(v) For a nontransportable sugar, the rate constants for the hello and recognition reactions appear to be similar. For D-maltose, Fig. 9 shows that the K_d for the hello reaction is 4.9 ± 1.9 mM, as compared to $K_d = 3.9 \pm 2.4$ mM for the recognition reaction. For the transportable D-glucose, the time constants for hello and recognition reactions are different, as shown in Fig. 3.

(vi) The relative importance of each exponential depends upon the temperature, as shown in Fig. 7 (middle), in which different exponentials dominate the time course at different temperatures.

The picture that emerges from our studies is of a very labile glucose transport protein. Virtually every interaction with a D-sugar results in a profound response, initially reflected in the large zero-time displacement and subsequently propagated through a series of two or three interrelated exponential reactions. Challenges applied to one face of the membrane are rapidly transmitted to conformational responses elicited on the other face. Although we have been able to make a rough description relating each of the exponentials to a specific function in the recognition/transport process, it is clear that the exponentials are interrelated. This is illustrated in Fig. 7 (middle) which shows how striking the effects of temperature are on the kinetic course of events, in which each exponential appears to be characterized by its own activation energy. The lability of the protein conformation and the interdependence of the reaction kinetics suggest that the neglect of any one kinetic step in a model system is fraught with danger. From this viewpoint, it is difficult to see how any reaction scheme can be constructed which does not take account of all the events. Subsequent simplifications will require strict justification of each approximation.

We should like to express our thanks to Michael R. Toon for his important contributions. This work was supported in part by a grant-in-aid from the American Heart Association, by the Squibb Institute for Medical Research and by The Council for Tobacco Research.

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Received 24 July 1992; revised 20 October 1992