Fluorine-19 NMR Studies of Glucosyl Fluoride Transport in Human Erythrocytes

Robert E. London and Scott A. Gabel
Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 USA

ABSTRACT Fluorine-19 magnetization transfer studies have been used to measure the transport rate of glucopyranosyl fluorides under equilibrium exchange conditions. Although rate constants and permeabilities could be determined for β-D-glucopyranosyl fluoride, the exchange rate for α-D-glucopyranosyl fluoride was found to be too slow for determination using this method. The time-dependent decomposition of the β-glucopyranosyl fluoride also limits the accuracy of the numerical results for this species; however, it is clear that the permeabilities of the α and β forms differ significantly, i.e., $P_\beta > P_\alpha$. This observation is in contrast to recent observations for *n*-fluoro-*n*-deoxyglucose, for which $P_\alpha > P_\beta$ for $n = 2$, 3, 4, or 6. The difference can be explained in terms of a simple alternating conformation model in which one of the conformations (with an external sugar-binding site) exhibits a preference for the β form of the molecule, while the second conformation (with an internal sugar binding site) exhibits a preference for the α form. Fluorine/hydroxyl substitutions unmask these preferences by selectively reducing the binding to one of the conformations, depending on the specific site of fluorination.

INTRODUCTION

The erythrocyte glucose transporter has served as a general model for transport by facilitated diffusion (Wheeler and Hinckle, 1985; Walmsley, 1988). Equilibrium exchange studies of the transport of fluorinated glucose analogs have recently been reported (Potts et al., 1990; Potts and Kuchel, 1992; O'Connell et al., 1994), which are made possible by the separation of the ^{19}F resonances corresponding to intracellular and extracellular compounds (London and Gabel, 1989). Such an approach is particularly useful for evaluating the anomeric dependence of the transport rates because separate resonances corresponding to the intra- and extracellular pools of each anomer are simultaneously observed. Furthermore, mutarotation rates are found to be completely negligible on the transport and spin-lattice relaxation time scales, and the presence of the fluorine substituent retards metabolic transformation of the fluorodeoxyglucose (O'Connell et al., 1994). Studies with fluorodeoxyglucose analogs in which the F/OH substitution is made at position 2, 3, 4, or 6 have shown that: 1) in all cases, the α anomer is transported more rapidly than the β; 2) the permeability ratio P_β/P_α is close to 1 for the 2- and 3-fluoro analogs, but significantly less than 1 for the 4- and 6-fluoro analogs; and 3) the 4-fluoro and 6-fluoro analogs also exhibit significantly lower permeabilities for either anomer than the 2- or 3-fluoro derivatives. These data were further interpreted in terms of a simple alternating conformation model for the erythrocyte glucose transporter.

Studies of the inhibition of L-sorbose entry into human erythrocytes by α- or β-glucopyranosyl fluoride have previously been reported (Barnett et al., 1973) and indicate that the β isomer has a significantly lower inhibition constant (K_I = 15.4 mM) compared with the α isomer (K_I = 78 mM). However, no studies of glucosyl fluoride transport appear to have been performed previously. Such studies are made somewhat more difficult by the chemical instability of β-glucosyl fluoride (Barnett, 1969; Konstantinidis and Sinnott, 1991). Nevertheless, it was found that the stability was sufficient to allow magnetization transfer measurements to be carried out, with small corrections introduced for the time-dependent decomposition. As discussed below, the transport behavior of α- and β-glucosyl fluoride can be viewed as an extrapolation of the trend exhibited by the fluorodeoxyglucose derivatives and can be interpreted in terms of an alterating conformation model for the carrier in which the two (inside or outside facing) conformations exhibit different anomeric preferences.

MATERIALS AND METHODS

α-D-Glucopyranosyl fluoride and β-D-glucopyranosyl fluoride were obtained from Sigma (St. Louis, MO). Proton-decoupled fluorine NMR experiments were carried out at 470.5 MHz on a GE GN-500 NMR spectrometer using a 5-mm ^{19}F {^1H} probe. Field homogeneity was optimized by shimming on the H_2O resonance, with a typical linewidth < 0.05 ppm. Blood samples were prepared using indated platelet-free human red blood cells obtained from the American Red Cross, as described prevously (O'Connell et al., 1994). Samples were not spun, to prevent centrifugation effects on the cells. The hematocrits of the samples were measured using capillary tubes spun on a microhematocrit centrifuge. 2,3,5,6-Tetrafluorophthalate was used as a concentration standard. This compound has a ^{19}F resonance that is 0.45 ppm upfield of the (extracellular) resonance of β-D-glucosyl fluoride, and the four equivalent fluorine nuclei give a single resonance.

Magnetization transfer studies were carried out as described previously (O'Connell et al., 1994) by selectively inverting the intra- and extracellular ^{19}F resonances of the glucosyl fluorides sequentially (Robinson et al., 1985) and monitoring the magnetization with a 90° pulse at delays of 0.001, 0.15, 0.30, 0.45, 0.60, and 0.75, and 20 s. The analysis of the data was based on the approach described by Perrin and Engler (1990) in which the data from the intra- and extracellular resonance inversion experiments

Received for publication 31 March 1995 and in final form 27 July 1995.
Address reprint requests to Dr. Robert E. London, Laboratory of Molecular Biophysics, NIEHS, MD 17-05, Box 12233, Research Triangle Park, NC 27709.

0006-3495/95/11/1814/00 $2.00

are organized into a series of 2 × 2 matrices, $\mathbf{M}(t_m)$; each matrix is then diagonalized, and the resulting elements are fit to a linear function of time, as described below:

$$\ln((\mathbf{M} - \mathbf{M}_{eq}) \cdot (\mathbf{M}_o - \mathbf{M}_{eq})^{-1}) = \mathbf{X}(\ln \Lambda)\mathbf{X}^{-1} = -t_m R \quad (1)$$

where $\mathbf{M}_o$ corresponds to the data matrix at $t_m = 0$, $\mathbf{M}_{eq}$ to the matrix of the fully relaxed system, and $\mathbf{X}$ is a square matrix of eigenvectors that transforms $(\mathbf{M} - \mathbf{M}_{eq}) \cdot (\mathbf{M}_o - \mathbf{M}_{eq})^{-1}$ into the diagonal matrix Λ. A linear fit of the data then yields the elements of the relaxation matrix, which are interpreted as shown below:

$$\begin{bmatrix} R_{11} & R_{12} \\ R_{21} & R_{22} \end{bmatrix} = \begin{bmatrix} 1/T_{1i} + k_{io} & -k_{oi} \\ -k_{io} & 1/T_{1o} + k_{oi} \end{bmatrix} \quad (2)$$

where k_{io} and k_{oi} are the apparent first-order rate constants for efflux and influx, and T_{1o} and T_{1i} are the spin lattice relaxation times for the extracellular and intracellular species, respectively.

RESULTS

A proton-decoupled ^{19}F spectrum of 5 mM α-glucopyranosyl fluoride and 5 mM β-glucopyranosyl fluoride in a suspension of human erythrocytes (H_t = 58%) is shown in Fig. 1. In contrast with the 2-, 3-, 4-, and 6-fluorodeoxyglucose (FDG) analogs, these two species do not interconvert, and hence are not true "anomeric forms" of the same molecule. As in the case of the *n*-fluoro-*n*-deoxy-D-glucose analogs studied previously (O'Connell et al., 1994), the resonances corresponding to the intracellular sugars are shifted downfield by 0.164 and 0.184 for the α and β fluorides, respectively. Hence, as with the 2-FDG and 3-FDG analogs, the β species experiences a larger downfield shift. Also consistent with previous observations, the intracellular resonances are significantly broader. This difference presumably reflects the larger intracellular viscosity and/or a combination of specific and nonspecific binding interactions with various intracellular molecules.

Although, as is apparent from Fig. 1, the α-glucosyl fluoride clearly enters the cell, the equilibrium exchange transport rate was found to be too slow at 37°C to measure by standard magnetization transfer techniques. In contrast, equilibrium exchange of the β-glucosyl fluoride proved to be amenable to study by magnetization transfer and was determined as described in Materials and Methods. A typical plot of the elements of the relaxation matrix as a function of time, obtained after linearization of the data by matrix diagonalization procedures, is shown in Fig. 2. As noted in Materials and Methods, the two diagonal elements that give positive slopes correspond to $R_{11} = 1/T_{1i} + k_{io}$ and $R_{22} = 1/T_{1o} + k_{oi}$, and the two off-diagonal elements with negative slopes correspond to $R_{12} = -k_{io}$ and $R_{21} = -k_{oi}$.

One important limitation on performing magnetization transfer studies on β-glucosyl fluoride is the instability of the molecule. Time-dependent measurements of resonance intensity carried out under the same conditions as the magnetization transfer studies were fit to a simple exponential decay function, and the decay rate for the β-glucopyranosyl fluoride was determined to be 0.45 h^{-1}. A time-dependent

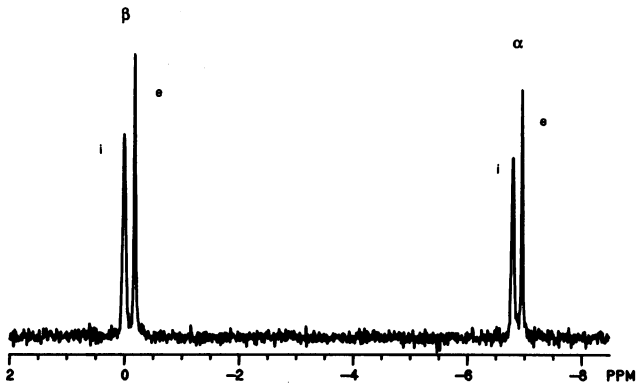

FIGURE 1 Proton decoupled ^{19}F spectrum (470.5 MHz) showing the resonances of α-D-glucopyranosyl fluoride and β-D-glucopyranosyl fluoride in the presence of human erythrocytes (H_t) suspended in 123 mM NaCl, 15 mM Tris/HEPES (pH 7.4), 5 mM ascorbate, T = 37°C.

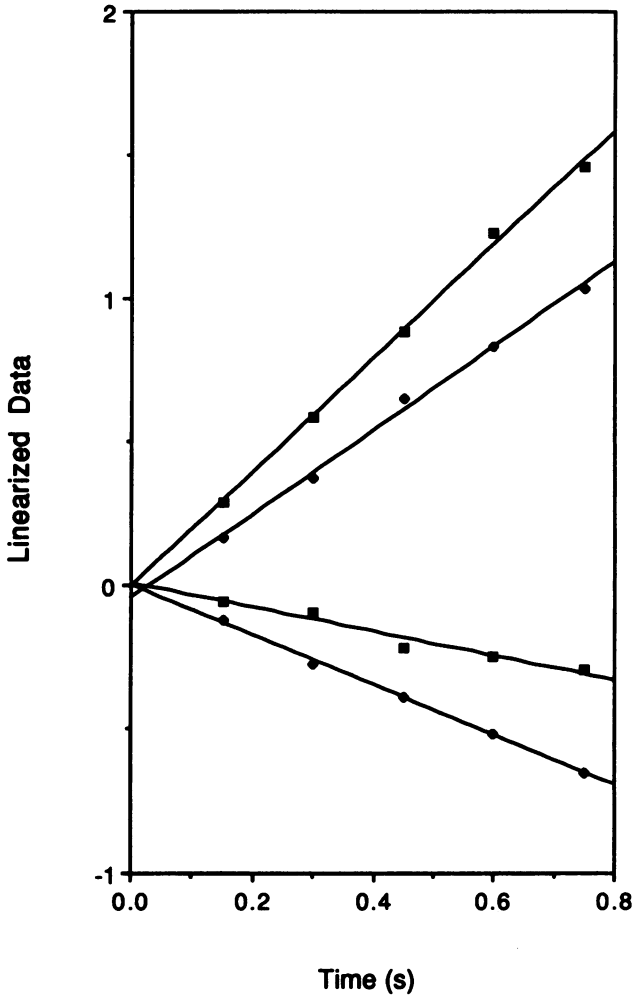

FIGURE 2 Linearized resonance intensity data derived as described by Eq. 1 for the transport of 10 mM β-D-glucopyranosyl fluoride, plotted as a function of the mixing time. Positive slopes: R_{11} (■), R_{22} (♦); negative slopes: R_{21} (■), R_{12} (♦).

correction factor was introduced into the data, although in general the effects on the computed efflux rate constants were small. A more significant and less easily corrected problem arises because of the competitive binding effects of the decomposition products, particularly glucose. For this reason, the results must be considered qualitatively rather than quantitatively.

As discussed previously (O'Connell et al., 1994), the inward and outward permeability coefficients, P_1 and P_{-1}, are equal in an equilibrium exchange experiment and can be calculated from the measured efflux rate constant using the relation (Kirk, 1990; Potts et al., 1990)

$$P_{-1} = \frac{V_i}{A} k_{-1} = \frac{\mathrm{MCV} f_w}{A_{cell}} k_{-1} \quad (3)$$

where f_w is the fraction of intracellular volume accessible to solute; V_i is the intracellular volume per milliliter, which is equal to $V_o H_t$; H_t is the hematocrit, A is the total surface area ($= A_{cell} H_t/\mathrm{MCV}$); A_{cell} ($= 1.43 \times 10^{-6}$ cm^2) is the surface area per cell (Sha'afi et al., 1967); and MCV is the mean cell volume, which equals 85 fl for red cells in isotonic solution (Chapman and Kuchel, 1990). The glucose efflux velocity was described using a Michaelis-Menten kinetic scheme:

$$v_{eff} = k_{-1}[\mathrm{glu}]_i = \frac{V_{max}^{ee}[\mathrm{glu}]_i}{K_m^{ee} + [\mathrm{glu}]_i} \quad (4)$$

where it is convenient to use the efflux rather than the influx rate constants, since otherwise the Michaelis parameters will be functions of the hematocrit. The permeability coefficient can then be expressed as

$$P_{-1} = \frac{V_i}{A} k_{-1} = \frac{V_i}{A} \frac{V_{max}^{ee}}{K_m^{ee} + [c]} = \frac{\mathrm{MCV} f_w}{A_{cell}} \frac{V_{max}^{ee}}{K_m^{ee} + [c]} \quad (5)$$

where K_m^{ee} and V_{max}^{ee} are the Michaelis parameters corresponding to the equilibrium exchange conditions of the study, and the results are shown in Fig. 3. The resulting K_m^{ee} (5.8 mM) is considerably smaller than the inhibition constant K_I (15 mM) obtained previously in studies of the inhibition of L-sorbose entry into erythrocytes by β-D-glucosyl fluoride at 25°C (Barnett et al., 1973) and is smaller than the K_α^{ee} and K_β^{ee} values of 8.3 mM and 7.0 mM obtained previously for the α and β anomers of 3-FDG (Potts and Kuchel, 1992). We believe that the low value obtained from the present studies reflects a systematic error due to the decomposition of the β-D-glucosyl fluoride. In particular, at lower concentrations the levels of breakdown products are well below their K_m values and hence exert only a minor perturbation on the results. At higher β-D-glucosyl fluoride concentrations the competitive binding by breakdown products becomes more significant and hence artificially reduces the observed permeability of the fluorinated sugar. However, despite this limitation on the accuracy of P_β, it is clear that $P_\beta > P_\alpha$ for the β- and α-D-glucosyl fluoride studies. The failure to observe by magnetization transfer an equilibrium exchange of α-D-glucosyl fluoride is consistent with the considerably higher

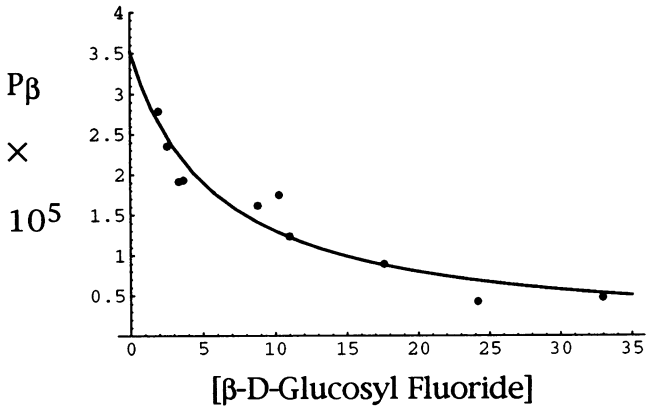

FIGURE 3 Plot of the permeability coefficient $10^5 \times P_\beta$ (in cm/s) for β-D-glucopyranosyl fluoride as a function of concentration (mM). Permeabilities were calculated from the efflux rate constants, which were obtained from the relaxation matrix determination. Data were fit to Eq. 5 using a nonlinear least-squares fitting capability of the commercial program, Mathematica. The plotted fit corresponds to $K_m^{ee} = 5.8$ mM; $(V_i/A) * V_{max}^{ee} = 2.1 \times 10^{-4}$ mM·cm/s, or $V_{max}^{ee} = 4.8$ mM/s.

$K_I = 78$ mM obtained for this species in the transport inhibition study noted above (Barnett et al., 1973).

Kinetic analysis and conformational implications

To the extent that the α- and β-glucosyl fluorides are structurally analogous to the corresponding anomeric forms, the present results indicate a reversal of the trend previously observed for the fluorodeoxyglucose derivatives (Potts and Kuchel, 1992; O'Connell et al., 1994); thus, $P_\beta > P_\alpha$ for glucosyl fluoride, and $P_\alpha > P_\beta$ for 2-, 3-, 4-, and 6-fluorodeoxyglucose. Replacement of the C-1 hydroxyl group by a fluorine could in principle replace a positive hydrogen bonding interaction with the C-1 OH acting as a hydrogen bond donor, with a repulsive interaction involving the electronegative fluorine substituent. However, the inhibition studies of Barnett et al. (1973) suggest that this is not the case, because the K_I values for α- and β-glucosyl fluoride are equal to or are one-fifth the value for 1-deoxy-D-glucose, respectively. Hence, the fluorine substituent on the β-D-glucosyl fluoride appears to enhance the interaction with the carrier relative to interaction of a hydrogen at the C-1 position. In contrast, the L-sorbose influx inhibition data obtained by Barnett et al. (1973) indicate that although the substitution of a β-fluorine for a hydrogen decreases K_I, the substitution of a β-fluorine for a C-1 hydroxyl increases K_I. As discussed below, the data obtained here can be interpreted in the context of a simple alternating carrier model subject to the assumptions that the outer-facing carrier favors the β-glucosyl fluoride over the α-glucosyl fluoride, and that the F/OH substitution weakens binding to the carrier in the outward-facing conformation relative to that in the inward-facing conformation.

Previous equilibrium exchange studies of glucose transport by NMR have utilized the kinetic scheme shown in Fig. 4 for transport of two competing substrates by a simple

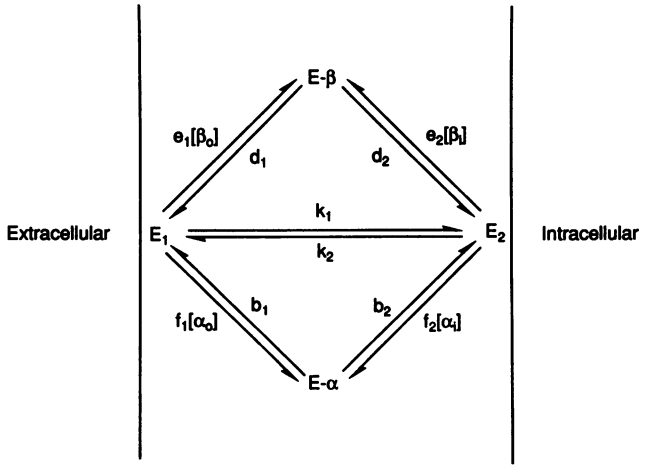

FIGURE 4 Kinetic model for the competing transport of two anomers via an alternating conformation model of the carrier, showing the 10 rate constants involved.

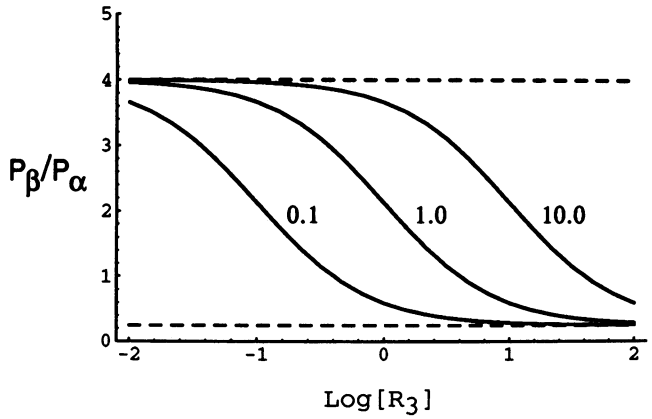

FIGURE 5 Theoretical plot of P_β/P_α for the model of Fig. 4 defined by Eq. 6. The dotted lines correspond to $P_\beta/P_\alpha = R_1 = e_1/f_1 = 4.0$, and to $P_\beta/P_\alpha = R_2 = e_2/f_2 = 0.25$. The three curves shown correspond to the $R_4 = 0.1$, 1.0, and 10.0, as indicated.

carrier (Barnett et al., 1975). Although the α- and β-glucosyl fluorides do not have to be treated as competing substrates because they do not interconvert and can be studied separately, this formalism is convenient for the present discussion. As discussed previously, the ratio of permeability coefficients obtained for two anomers measured simultaneously can be expressed in terms of four ratios of the rate constants shown in Fig. 4:

$$\frac{P^\beta}{P^\alpha} = \frac{k_1^\beta}{k_1^\alpha} = \frac{R_1R_4 + R_2R_3}{R_3 + R_4} \tag{6}$$

where $R_1 = e_1/f_1$ is the ratio of association constants for β/α-glucose anomers with the carrier on side 1 of the membrane, $R_2 = e_2/f_2$ is the corresponding ratio on side 2 of the membrane, $R_3 = e_1/e_2$ is the ratio of the association constant for the β anomer with the carrier on side 1 relative to that on side 2, and finally $R_4 = k_1/k_2$ reflects the distribution of the unloaded carrier between the two sides of the membrane. From Eq. 6 above, we note that if $R_1 = R_2$, i.e., the binding ratio on both sides of the membrane is the same, then the observed permeability ratio equals R_1, independently of parameters R_3 and R_4. Alternatively, if the association rate constants for the two species differ for the inward- and outward-facing states of the carrier, Eq. 6 predicts that P_β/P_α considered as a function of R_3 will have a sigmoidal shape varying from limits R_1 for $R_3 \ll 1$ to R_2 for $R_3 \gg 1$. This general behavior is illustrated in Fig. 5 for the specific case of $R_1 = 4$, $R_2 = 0.25$. This behavior arises because in the equilibrium exchange experiment, the observed exchange rates will be dominated by slower steps in the process. Hence, poorer association on side 1 of the membrane ($R_3 \ll 1$) leads to an observed permeability coefficient ratio characteristic of side 1 of the membrane.

We propose that this forms an attractive basis for considering the effects of F/OH substitution. In particular, as discussed previously, replacement of a hydroxyl group by a fluorine can be considered to introduce either a global or a local perturbation. In the first case, all of the kinetic rate constants will be altered and no further analysis is possible. In the case of the fluorodeoxyglucose analogs, it is attractive to consider the perturbation as primarily a local effect, so that the F/OH substitution at some position other than C-1 will exert a minimal perturbation on the parameters R_1 or R_2, which reflect the ratio of affinities for the α and β anomeric forms, but will more significantly perturb R_3, i.e., the relative affinity of the molecule for the two states of the carrier. In this case, the position dependence of the observed P_β/P_α ratio corresponds to moving along the sigmoidal curve of Fig. 5. The substitutions at positions 4 and 6 reduce the affinity to side 2 (intracellular) of the membrane, R_3 becomes >1, and the observed P_β/P_α ratio approaches R_2. Thus, it is concluded that in state 2 the carrier has an inherent preference for the α anomeric form of the sugars. Alternatively, the P_β/P_α ratios near 1 observed for 2- and 3-FDG must then be reflecting near-cancellation of the α anomeric preference of side 2 and the β preference of side 1.

Extending this analysis to the case of glucosyl fluoride, the F/OH sbustitution at the C-1 position weakens binding to side 1 of the membrane so that R_3 becomes <1. In this case, the observed P_β/P_α ratio approaches R_1, which is characteristic of the carrier in state 1 (extracellular glucose binding site). Hence, based on the above line of reasoning, the erythrocyte glucose transporter has an inherent preference for the β anomeric form when it is in state 1, and an inherent preference for the α anomeric form when it is in state 2.

This type of asymmetric carrier conformational model represents an extension of the model proposed by Barnett et al. (1975) on the basis of selective inhibition studies. According to that model, state 1 of the carrier, which has the binding site facing outward, has C-4 and C-6 in contact with the solvent (Fig. 6, *A* and *C*), whereas state 2 of the carrier, which has an inward-facing sugar-binding site, has C-1 in contact with the solvent. Based on this analogy, we can identify state 1 of the carrier, which binds extracellular glucose, as the state exhibiting a preference for the β

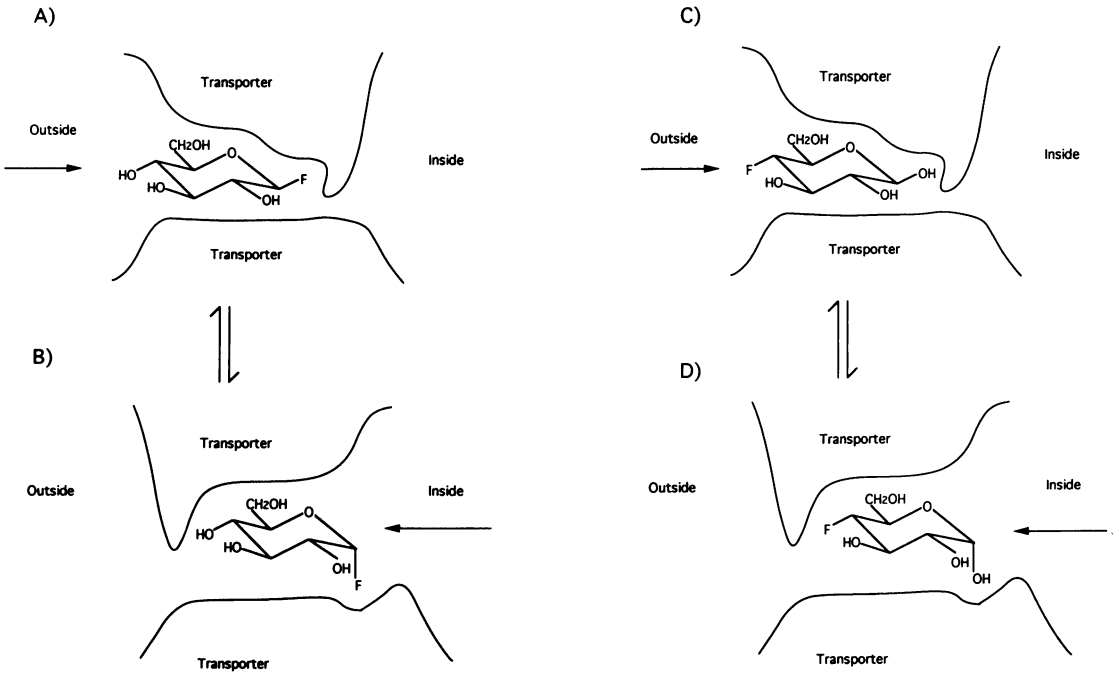

FIGURE 6 Structural model illustrating the interaction of glucosyl fluoride (*A* and *B*) or 4-fluoro-4-deoxyglucose (*C* and *D*) with the carrier. The basic alternating conformation model is extended in order to exhibit a preference for the α species when the glucose binding site is on the inner side of the membrane (*B* and *D*), and a preference for the β species when the glucose binding site is on the outside of the membrane (*A* and *C*). Of course, the conformational change is not accompanied by an $\alpha \leftrightarrow \beta$ interconversion; the isomeric form has been chosen to illustrate the structural preference of the transporter in the two conformational states.

isomer, and state 2 of the carrier, which binds intracellular glucose, as the state exhibiting a preference for the α isomer. It is easy to conclude that state 1, with an externally oriented binding site, might exhibit a strong isomer preference, because it interacts with the C-1 end of the glucose molecule. It is somewhat harder to interpret an isomer preference of state 2, because in this state the C-1 end of the molecule is in contact with the solvent. In this case, some type of intermediate structure might be proposed, as illustrated in Fig. 6, *B* and *D*, in which the glucose bound to state 2 is not completely enclosed by the carrier.

The behavior outlined above is analogous to the kinetic behavior of a two-step catalytic process, in which the features of the slower step dominate the observed specificity. In this case, fluorine substitution at the C-1 position slows down the association with the carrier when it has an extracellular-facing binding site, so that the preferences of this state are unmasked. Alternatively, F/OH substitution at C-4 and C-6 slows down the association rate with the intracellular-facing carrier, so that the anomeric preferences of this state become dominant.

Helpful discussions with Dr. Arron Xu are gratefully acknowledged.

REFERENCES

Barnett, J. E. G. 1969. Acid and alkaline hydrolysis of glycopyranosyl fluorides. *Carbohydr. Res.* 9:21–31.

Barnett, J. E. G., G. D. Holman, R. Holman, R. A. Chalkley, and K. A. Munday. 1975. Evidence for two asymmetric conformational states in the human erythrocyte sugar-transport system. *Biochem. J.* 145:417–429.

Barnett, J. E. G., G. D. Holman, and K. A. Munday. 1973. Structural requirements for binding to the sugar-transport system of the human erythrocyte. *Biochem. J.* 131:211–221.

Chapman, B. E., and P. W. Kuchel. 1990. Fluoride transmembrane exchange in human erythrocytes measured with ^{19}F NMR magnetization transfer. *Eur. J. Biophys.* 19:41–45.

Kirk, K. 1990. NMR methods for measuring membrane transport rates. *NMR Biomed.* 3:1–16.

Konstantinidis, A., and M. L. Sinnott. 1991. The interaction of 1-fluoro-D-glucopyranosyl fluoride with glucosidases. *Biochem. J.* 279:587–593.

London, R. E., and S. A. Gabel. 1989. Determination of membrane potential and cell volume by ^{19}F NMR using trifluoroacetate and trifluoroacetamide probes. *Biochemistry.* 33:2378–2382.

O'Connell, T. M., S. A. Gabel, and R. E. London. 1994. Anomeric dependence of fluorodeoxyglucose transport in human erythrocytes. *Biochemistry.* 33:10985–10992.

Perrin, C. L., and R. E. Engler. 1990. Weighted linear-least-squares analysis of EXSY data from multiple 1D selective inversion experiments. *J. Magn. Reson.* 90:363–369.

Potts, J. R., A. M. Hounslow, and P. W. Kuchel. 1990. Exchange of fluorinated glucose across the red-cell membrane measured by ^{19}F-n.m.r. magnetization transfer. *Biochem. J.* 266:925–928.

Potts, J. R., and P. W. Kuchel. 1992. Anomeric preference of fluoroglucose exchange across human red-cell membranes. *Biochem. J.* 281:753–759.

Robinson, G., P. W. Kuchel, B. E. Chapman, D. M. Doddrell, and M. G. Irving. 1985. A simple procedure for selective inversion of NMR resonances for spin-transfer enzyme kinetic measurements. *J. Magn. Reson.* 63:314–319.

Sha'afi, R. I., G. T. Rich, V. W. Sidel, W. Bossert, and A. K. Solomon. 1967. The effect of the unstirred layer on human red cell water permeability. *J. Gen. Physiol.* 50:1377–1399.

Walmsley, A. R. 1988. The dynamics of the glucose transporter. *Trends Biochem. Sci.* 13:226–231.

Wheeler, T. J., and P. C. Hinkle. 1985. The glucose transporter of mammalian cells. *Annu. Rev. Physiol.* 47:503–517.