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Anomeric preference of fluoroglucose exchange across human red-cell membranes

^{19}F-n.m.r. studies

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The rates of exchange across the human red-cell membrane of the α- and β-anomers of the glucose derivative 3-fluoro-3-deoxy-D-glucose (3FG) were measured, under equilibrium-exchange conditions, using a ^{19}F-n.m.r.-magnetization-exchange procedure. In experiments carried out over a range of 3FG concentrations (3.4–113 mM), the α-anomer was found to be transported with a smaller K_m (greater apparent affinity) than the β-anomer. In two experiments carried out at 34 and 37 °C the ratio (α/β) of the Michaelis constants for exchange was 0.75 ± 0.07 and 0.83 ± 0.07 respectively and the $V_{max.}$ for 3FG exchange was 28 ± 3 and 33 ± 3 mmol·s^{-1}·litre of cells^{-1} respectively. In several experiments carried out at a single 3FG concentration (17 mM) and at 37 °C, using red cells from four individuals, the rate of exchange of the α-anomer across the membrane was significantly higher than that of the β-anomer. The weighted mean value of the above-mentioned ratio was 0.79 ± 0.07 for the four donors.

INTRODUCTION

The equilibration of glucose across the human red-cell membrane is catalysed by an integral membrane protein with an apparent molecular mass of 55 kDa [1]. It has been suggested that the binding of glucose to the red-cell glucose transporter involves hydrogen-bonding between protein hydrogen atoms and the sugar hydroxy oxygen atoms at C-1, C-3, probably C-4 and possibly C-6 [2]. Since the α- and β-anomers of glucose differ in the orientation of the hydroxy group on C-1 (being axial or equatorial to the plane of the pyranose ring respectively), it seems likely that the two anomers would bind to the transport protein with different affinity; the question remains as to whether this difference in binding affinity would be sufficient to lead to a difference in the rate of transport.

Of the many studies that have been carried out on the kinetics of glucose transport across the red-cell membrane (for a review, see [3]), relatively few have examined the question of the relative rates of transport of the two anomers. This is despite the suggestion that the failure to consider the substrate D-glucose as a *mixture* of two substrates, α- and β-D-glucose, may account in part for some inconsistencies of the observed kinetics with the proposed alternating conformer or 'one-site' model of the mechanism of glucose exchange [4].

In previous work, the anomeric preference of red-cell glucose transport has been studied using both analyses of substrate binding and, the measurement of exchange rates. The results of Reinwein and co-workers [5], LeFevre & Marshall [6] and Barnett and co-workers [2] indicated that the β-anomer of glucose bound to the carrier with greater affinity than the α-anomer. Faust [7] observed that the β-anomer was transported almost three times more quickly than the α-anomer, whereas Fujii and co-workers [8] reported that the ratio of the rates of exchange of the anomers (β/α) was 1.13:1. In studies of the interconversion of the intracellular- and extracellular-facing conformations of the purified human red-cell glucose-transport protein, Appleman & Lienhard [9] observed that the α-anomer of glucose was more 'effective' than the β-anomer in speeding the interconversion.

In the present work we studied the anomeric preference of exchange of 3-fluoro-3-deoxy-D-glucose (3FG) across the human red-cell membrane under equilibrium-exchange conditions at (or close to) physiological temperatures. We used an equilibrium mixture of the two anomers rather than the purified anomer preparations that have been used in most studies of the anomeric preference of glucose exchange. From the permeability coefficients for α- and β-3FG exchange measured over a range of 3FG concentrations, we calculated the anomeric preference of exchange at each 3FG concentration. From the dependence of the permeability coefficients on the 3FG concentration, we obtained estimates of the K_m and $V_{max.}$ for the exchange of α- and β-3FG across the red-cell membrane. We also determined the anomeric preference of exchange in red cells from four donors at a single (total) 3FG concentration.

MATERIALS AND METHODS

Materials

3FG was from Sigma, St. Louis, MO, U.S.A. ^2H_2O (99.75 atom %) was from the Australian Institute of Nuclear Science and Engineering, Lucas Heights, N.S.W., Australia. All other reagents were of A.R. grade.

Red cells

Blood (usually 20–30 ml) was obtained by venipuncture from healthy donors in our laboratory and diluted to 50 ml with ice-cold saline (0.154 M-NaCl). Cell suspensions were either prepared immediately or cells were washed twice in ice-cold saline before being stored overnight in 8 mM-glucose at 5 °C. Erythrocyte suspensions were prepared, before the n.m.r. experiments, as follows. The cells were washed four times in ice-cold saline, then suspended in phosphate-buffered saline (PBS; 140 mM-NaCl/20 mM-phosphate, pH 7.4, at 20 °C), bubbled with CO (5 min, 5 °C), collected by centrifugation, and then washed twice in PBS/10 mM-inosine (prebubbled with CO) or in PBS/8 mM-glucose (prebubbled with CO). The PBS/inosine was used in the experiments in which the permeability coefficients were measured over a range of 3FG concentrations to avoid complicating the

Abbreviations used: 3FG, 3-fluoro-3-deoxy-D-glucose; PBS, phosphate-buffered saline (composition and pH given in the text); Ht, haematocrit.

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kinetic analysis with the presence of another substrate for the glucose-transport protein. Inosine can be metabolized by the cells, entering glycolysis via the pentose phosphate pathway [10]. Cell suspensions were prepared by combining packed cells with PBS/10 mM-inosine or PBS/8 mM-glucose to give an haematocrit (Ht) of ~ 0.4.

After addition of 3FG, the cell suspensions were incubated at 37 °C for at least 30 min (depending on the 3FG concentration) to allow full transmembrane equilibration of 3FG and glucose. The cell suspensions were then stored at 5 °C until required for the n.m.r. experiment, when they were again incubated at 37 °C for ~ 15 min before being placed in the n.m.r. spectrometer. The Ht of each cell suspension was measured in duplicate. The number of cells/ml of suspension was determined in duplicate by using a Sysmex Microcellcounter CC-130 (Toa Medical Electronics Co., Kobe, Japan). The total solute-accessible volume in each cell suspension was calculated assuming that 0.717 of the intracellular volume was accessible to the 3FG molecules [11,12].

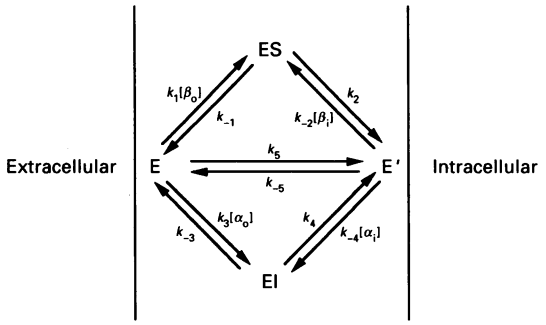
N.m.r.

As ^2H_2O has been shown to decrease the rate of transmembrane exchange of glucose in human red cells [13], field/frequency locking of the spectrometer was achieved by using an 'external' capillary filled with ^2H_2O placed coaxially in the sample. Broad-band proton-decoupled ^{19}F-n.m.r. spectra were acquired at 376.43 MHz in the Fourier-transform mode using a Bruker AMX400 spectrometer. The temperature of the sample in the magnet was measured using an ethylene glycol capillary [14]. Experiments were carried out at 37 °C unless otherwise stated. The 'over-determined' one-dimensional exchange analysis was performed in a manner similar to the ^{31}P-n.m.r. experiment that was described previously [15], but with the following n.m.r. parameters: for each spectrum 4–16 transients (depending on the concentration of 3FG used) were averaged and 12 s ($> 5 \times T_1$, where T_1 is the longitudinal relaxation time) were allowed between the acquisition of each transient; the $\pi/2$ acquisition pulse was ~ 22 μs; the spectral width was 5000 Hz, with the free-induction decay digitized into 12 k ('zero-filled' to 16 k) or 16 k data points and processed using 1 Hz of line-broadening; t_m (mixing time) was 0.5 s, four evolution times were used and the times were chosen (*a*) so as to provide optimal signal-to-noise ratios for both the intra- and extra-cellular resonances and (*b*) to minimize relaxation during the pulse sequence. The equilibrium intensities of the intra- and extra-cellular resonances in each sample (and the anomeric composition of 3FG) were determined from a 'fully-relaxed' spectrum acquired with the same n.m.r. parameters as those described above.

Saturation-transfer experiments were carried out as described previously [16], with the following changes: spectra from eight transients were acquired on a Bruker AMX400 spectrometer at 376.43 MHz; the $\pi/2$ acquisition pulse was 48 μs; 16 k data points were collected; and the spectral width was 8064 Hz.

Statistical methods

Statistical analysis of the data involved applications of Student's *t* test as described by Bailey [17].



Scheme 1. Chemical reaction scheme describing the carrier-mediated exchange of the α- and β-anomers of 3FG

α_o and β_o denote extracellular populations of α- and β-3FG respectively, whereas α_i and β_i represent the intracellular populations of α- and β-3FG respectively. E and E′ denote forms of the carrier with the binding site exposed at the extra- and intra-cellular sides of the membrane respectively. Exchange was measured at equilibrium, so $[\alpha_o] = [\alpha_i]$ and $[\beta_o] = [\beta_i]$.

THEORY

The simplest kinetic scheme which describes exchange of the *un*loaded carrier between the two faces of the membrane and also describes the exchange of one anomer of 3FG across the membrane in the presence of the other anomer (which will act as a competitive inhibitor of exchange) is shown in Scheme 1. This Scheme is based on the 'conventional carrier' model of glucose transport [3], but assumes that only a single carrier-substrate complex (i.e. ES or EI) exists. However, the form of the steady-state equations derived from the Scheme are identical with those obtained if two carrier-substrate complexes (ES and EP or EI and EQ) existed [18].

Expressions for the velocity of influx (v^β and v^α) of the two anomers were derived by using the method of King & Altman [19]. The Michaelis–Menten equations describing the velocity of influx of one anomer in the presence of the other anomer which acts as a competitive inhibitor are given below. When exchange of the β-anomer is measured:

$$v^\beta = \frac{V^\beta_{max.}[\beta_o]}{K^\beta + \frac{K^\beta}{K^\alpha}[\alpha_o] + [\beta_o]} \quad (1)$$

and when exchange of the α-anomer is measured:

$$v^\alpha = \frac{V^\alpha_{max.}[\alpha_o]}{K^\alpha + \frac{K^\alpha}{K^\beta}[\beta_o] + [\alpha_o]} \quad (2)$$

Thus we obtained expressions for the Michaelis constants and maximal velocity for the influx of each anomer measured in the presence of the other one:

$$K^\beta = \frac{[\beta_i](k_{-1}k_{-2}k_4 + k_{-1}k_{-2}k_{-3} + k_{-2}k_4k_5 + k_{-2}k_{-3}k_5) + [\alpha_i](k_2k_{-3}k_{-4} + k_{-1}k_{-3}k_{-4} + k_2k_{-4}k_5 + k_{-1}k_{-4}k_5) + k_{-1}k_4k_{-5} + k_2k_{-3}k_{-5} + k_{-1}k_{-3}k_{-5} + k_2k_4k_{-5} + k_2k_4k_5 + k_{-1}k_4k_5 + k_2k_{-3}k_5 + k_{-1}k_{-3}k_5}{[\alpha_i](k_1k_{-3}k_{-4} + k_1k_2k_{-4}) + [\beta_i](k_1k_{-2}k_4 + k_1k_{-2}k_{-3}) + k_1k_4k_{-5} + k_1k_{-3}k_{-5} + k_1k_2k_4 + k_1k_2k_{-3}} \quad (3)$$

$$K^\alpha = \frac{[\beta_i](k_{-1}k_{-2}k_4 + k_{-1}k_{-2}k_{-3} + k_{-2}k_4k_5 + k_{-2}k_{-3}k_5) + [\alpha_i](k_2k_{-3}k_{-4} + k_{-1}k_{-3}k_{-4} + k_2k_{-4}k_5 + k_{-1}k_{-4}k_5) + k_{-1}k_4k_{-5} + k_2k_{-3}k_{-5} + k_{-1}k_{-3}k_{-5} + k_2k_4k_{-5} + k_2k_4k_5 + k_{-1}k_4k_5 + k_2k_{-3}k_5 + k_{-1}k_{-3}k_5}{[\alpha_i](k_{-1}k_3k_{-4} + k_2k_3k_{-4}) + [\beta_i](k_{-1}k_{-2}k_3 + k_{-2}k_3k_4) + k_2k_3k_{-5} + k_{-1}k_3k_{-5} + k_{-1}k_3k_4 + k_2k_3k_4} \quad (4)$$

$$V^{\beta}_{max.} = \frac{(k_{-3}k_{-4}[\alpha_i] + k_4k_{-5} + k_{-3}k_{-5})k_2[E_t]}{[\alpha_i](k_{-3}k_{-4} + k_2k_{-4}) + [\beta_i](k_{-2}k_4 + k_{-2}k_{-3}) + k_4k_{-5} + k_{-3}k_{-5} + k_2k_4 + k_2k_{-3}} \quad (5)$$

$$V^{\alpha}_{max.} = \frac{(k_{-1}k_{-2}[\beta_i] + k_2k_{-5} + k_{-1}k_{-5})k_4[E_t]}{[\alpha_i](k_{-1}k_{-4} + k_2k_{-4}) + [\beta_i](k_{-1}k_{-2} + k_{-2}k_4) + k_2k_{-5} + k_{-1}k_{-5} + k_{-1}k_4 + k_2k_4} \quad (6)$$

In eqns. (1–6), K^{β} and K^{α} are the Michaelis constants for influx, under equilibrium-exchange conditions, of the β- and α-anomers respectively. $V^{\beta}_{max.}$ and $V^{\alpha}_{max.}$ are the maximal velocities for these two exchange reactions; $[\alpha_o]$ and $[\alpha_i]$ denote the extra- and intra-cellular concentrations respectively of α-3FG; and $[\beta_o]$ and $[\beta_i]$ denote the extra- and intra-cellular concentrations respectively of β-3FG; $[E_t]$ is the combined (total) concentration of the enzyme species. It can be shown that the expression for K^{β}, and thus its value, is the same in eqns. (1) and (2) and, similarly, that the value of K^{α} is the same in eqns. (1) and (2).

N.m.r.-magnetization-transfer analyses, including the 'over-determined' one-dimensional exchange analysis, in which first-order rate constants for equilibrium exchange are measured, can be viewed as 'tracer' exchange experiments [20,21]. Accordingly, the first-order influx rate constants, k^{β}_1 and k^{α}_1, can be obtained from eqns. (1) and (2) by dividing each by $[\beta_o]$ and $[\alpha_o]$ respectively. That is, the tracer exchange velocities are given by: $v^{\beta} = k^{\beta}_1 \times [\beta_o]$ and $v^{\alpha} = k^{\alpha}_1 \times [\alpha_o]$. The first-order rate constants are measured under equilibrium-exchange conditions, so, in eqns. (1) and (2), $[\beta_o] = [\beta_i] = [\beta]$ and $[\alpha_o] = [\alpha_i] = [\alpha]$. Note also that in our experiments (under the given experimental conditions) the ratio of the concentrations of the α- and β-anomers is constant (i.e., $[\alpha] = a[\beta]$, where $a = 0.85 \pm 0.02$; see Fig. 1 and the Results section).

Thus, from eqns. (1) and (2):

$$k^{\beta}_1 = \frac{V^{\beta}_{max.}}{K^{\beta} + \frac{K^{\beta}}{K^{\alpha}}[\alpha] + [\beta]} \quad (7)$$

and

$$k^{\alpha}_1 = \frac{V^{\alpha}_{max.}}{K^{\alpha} + \frac{K^{\alpha}}{K^{\beta}}[\beta] + [\alpha]} \quad (8)$$

Using the observation of Krupka [22] that the specificity of the human red-cell glucose transporter for various monosaccharide substrates is expressed in the affinity of the transporter for the sugar rather than in the maximal velocity of exchange, we made the assumption that the $V_{max.}$ for the transport of α- and β-3FG was the same. Thus $V^{\alpha}_{max.} = V^{\beta}_{max.}$, and it transpires that an estimate of the relative *apparent* affinity of exchange of the two anomers is obtained by calculating the ratio of the first-order rate constants:

$$\frac{k^{\beta}_1}{k^{\alpha}_1} = \frac{K^{\alpha}}{K^{\beta}} \quad (9)$$

In the experiments carried out using red cells from a number of different donors, glucose was also present in the cell suspension. In this case, eqns. (1) and (2), would have to be modified to account for the presence of a second competitive inhibitor of the exchange of β- or α-3FG. However, if glucose acts as a purely competitive inhibitor, then eqn. (9) is still valid, and the anomeric preference of 3FG exchange can be calculated from the ratio of the first-order rate constants measured for the two anomers of 3FG.

The rate of 3FG exchange across the red-cell membrane was measured using ^{19}F-n.m.r. and the 'over-determined' one-dimensional exchange analysis [23]. A typical set of spectra is shown in Fig. 1. The first-order influx rate constants (k^{β}_1 and k^{α}_1) that were measured are dependent on the Ht of the cell suspension; therefore, for the following analysis, the rate constants were converted into membrane permeability coefficients (P^{β} and P^{α}). The permeability coefficient is independent of the Ht of the cell suspension, and was calculated from the k_1 for each anomer by using the following equation:

$$P = k_1 \frac{V^{out}}{A^{total}} \quad (10)$$

where, V^{out} is the volume of the extracellular compartment/ml of cell suspension, as calculated from the Ht of the cell suspension ($V^{out} = 1 - Ht$), and A^{total} is the total red-cell-membrane area/ml of cell suspension, which was calculated from the cell count and the mean surface area of the human red-cell membrane [$(1.43 \pm 0.08) \times 10^{-6}$ cm^2; [24]). Thus eqn. (9) becomes:

$$\frac{P^{\beta}}{P^{\alpha}} = \frac{K^{\alpha}}{K^{\beta}} \quad (11)$$

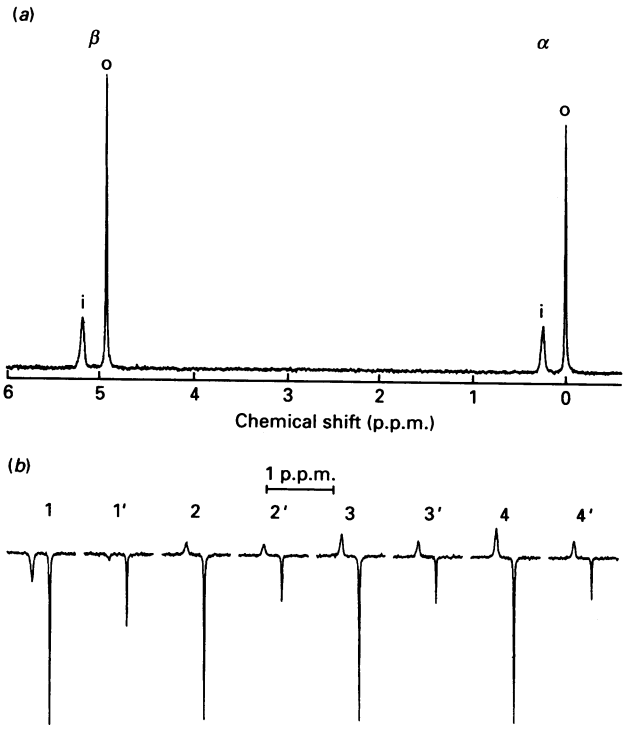


Fig. 1. 'Over-determined' one-dimensional exchange analysis

(*a*) A fully-relaxed ^{19}F-n.m.r. spectrum of 3FG (17 mM) in a suspension of human red cells (Ht = 0.40). For each of the β- and α-anomers there is an intra- (i) and extra- (o) cellular population. The spectrum was acquired with 16 transients and an inter-transient delay of 12 s. Other n.m.r. parameters are given in the Materials and methods section. (*b*) Four pairs of spectra (β-anomer region of spectrum only). Each pair arises from a single evolution time with 0 s (unprimed number above spectrum) and 0.5 s (primed number above spectrum) mixing times. Evolution times were: 1 and 1', 3 μs; 2 and 2', $5/(16 \times \delta\nu)$ s; 3 and 3', $6/(16 \times \delta\nu)$ s and 4 and 4', $7/(16 \times \delta\nu)$ s, where $\delta\nu$ was the separation (in Hz) between the intra- and extra-cellular β-3FG resonances. Each spectrum was acquired with eight transients and an inter-transient delay of 12 s.

RESULTS

Anomeric ratio

The equilibrium anomeric composition of 3FG, determined from fully-relaxed ^{19}F-n.m.r. spectra of 3FG (3.4–113 mM) in cell suspensions, was $0.54\pm0.01:0.46\pm0.01$ (β-3FG/α-3FG). This ratio was used to calculate the concentration of each anomer from the known total 3FG concentration.

Anomeric preference of exchange over a range of 3FG concentrations

Table 1 shows the results of experiments in which the permeability coefficients for 3FG exchange were measured at 37 °C, over a range of 3FG concentrations, using the 'over-determined' one-dimensional exchange analysis. At each 3FG (total) concentration, the anomeric preference of 3FG exchange was calculated from the permeability coefficients for the two anomers measured in the same sample. In two experiments (on red cells obtained from the same donor), one performed at 34 °C and the other at 37 °C (Table 1) the weighted mean anomeric preference over the range of 3FG concentrations was 0.75 ± 0.07 and 0.83 ± 0.07 respectively. These results indicate that the α-anomer was transported across the membrane with greater apparent affinity than the β-anomer.

Steady-state rate parameter values

The substrate-concentration-dependence of P^β and P^α was used to obtain estimates of K^β, K^α and $V_{max.}$ for 3FG exchange. Eqns. (7) and (8) (using eqn. 10) can be written in the following form:

$$P^\beta = \frac{\dfrac{V^\beta_{max.}V^{out}}{\left(\dfrac{aK^\beta}{K^\alpha}+1\right)A^{total}}}{\dfrac{K^\beta}{\left(\dfrac{aK^\beta}{K^\alpha}+1\right)}+[\beta]} \equiv \frac{A^\beta}{B^\beta+[\beta]} \qquad (12)$$

Table 1. Anomeric preference of 3FG exchange over a range of concentrations

The permeability coefficients (P^β and P^α) for 3FG exchange were measured at 37 °C using the 'over-determined' one-dimensional exchange analysis over a range of substrate concentrations. At each total 3FG concentration the concentration of each anomer was calculated by assuming that $[\alpha\text{-3FG}] = (0.85\pm0.02)\times[\beta\text{-3FG}]$. Unless otherwise stated, the permeability coefficient is the weighted mean of two determinations, and the S.D. is the weighted S.D. calculated from the S.D. of the individual permeability coefficients.

[β-3FG] (mM)	P^β ($10^5\times P$; cm·s^{-1})	[α-3FG] (mM)	P^α ($10^5\times P$; cm·s^{-1})	P^β/P^α
1.8	9.9±0.6	1.6	11.1±0.7	0.90±0.08
5.5	5.8±0.4	4.7	7.3±0.5	0.81±0.07
7.3	4.7±0.2	6.3	5.6±0.3	0.84±0.06
9.1	4.4±0.3	7.8	5.4±0.3	0.81±0.07
*11	4.2±0.3	*9.4	5.1±0.3	0.82±0.07
15	2.9±0.2	13	3.6±0.2	0.81±0.07
*31	1.6±0.1	*26	2.3±0.1	0.69±0.06
61	0.82±0.05	52	1.1±0.1	0.73±0.07
		Weighted mean ± weighted S.D....		0.83±0.07

* Single measurement.

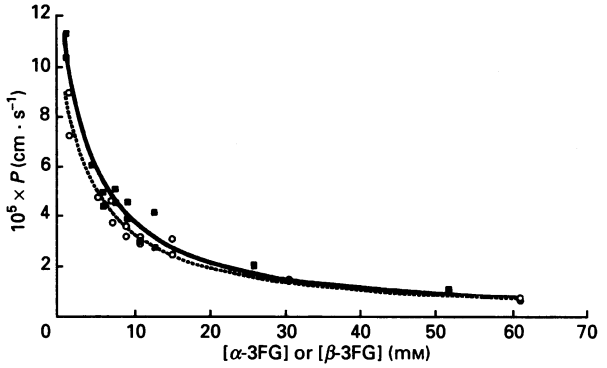


Fig. 2. Dependence of the red-cell permeability coefficients for α- and β-3FG on the concentration of each anomer

Permeability coefficients (P) were measured for both α- (■) and β-3FG (○) at 34 °C. Red-cell preparation was as described in the Materials and methods section. The lines through the α-anomer data (continuous line) and β-anomer data (broken line) were fitted by non-linear least-squares regression of eqns. (13) and (12) respectively on to the data. The fitting parameters A^β and A^α were $(4.8\pm0.4)\times10^{-4}$ mM·s^{-1}·cm and $(5.1\pm0.4)\times10^{-4}$ mM·s^{-1}·cm respectively. B^β and B^α were 4.1 ± 0.6 mM and 3.2 ± 0.4 mM respectively.

and

$$P^\alpha = \frac{\dfrac{V^\alpha_{max.}V^{out}}{\left(\dfrac{K^\alpha}{aK^\beta}+1\right)A^{total}}}{\dfrac{K^\alpha}{\left(\dfrac{K^\alpha}{aK^\beta}+1\right)}+[\alpha]} \equiv \frac{A^\alpha}{B^\alpha+[\alpha]} \qquad (13)$$

where V^{out} is the extracellular volume/ml of suspension, A^{total} is the red-cell-membrane area/ml of suspension (see above eqn. 11), and the values of A and B for each anomer are composite parameters defined by eqns. (12) and (13); the other parameters are defined above. By using non-linear least-squares regression, eqns. (12) and (13) were fitted to a graph of the permeability coefficients of the β- and α-anomers of 3FG respectively as a function of their concentration (Fig. 2). Having already made the assumption [22] that the maximal velocities of exchange for the two anomers are the same, K^α and K^β were calculated from the values of B determined from the data for the α- and β-anomers respectively. In each case the term in large parentheses in eqns. (12) and (13) was calculated using the weighted mean value for K^α/K^β calculated over a range of 3FG concentrations (as in Table 1) and a value of a of 0.85 ± 0.02. For the data obtained at 34 °C (Fig. 2), the values of K^α and K^β were 6.0 ± 0.9 mM and 8.8 ± 1.3 mM respectively. For the data obtained at 37 °C, the values of K^α and K^β were 8.3 ± 1.3 mM and 7.0 ± 1.0 mM. The large standard deviations in these data arise from the relatively large uncertainty in the values of B determined in the fit. Since B is an *apparent* K_m, it is more sensitive to the values of the permeability coefficients measured at the *lowest* substrate concentration, where the signal-to-noise ratios, in the n.m.r. spectra, are lowest. Thus a more precise estimate of the relative affinity with which the two anomers are transported across the red-cell membrane is obtained by determining the weighted mean of the relative affinities calculated at *each* concentration of 3FG, as is presented in Table 1. The values of $V_{max.}$ calculated from the data obtained at 34 °C and 37 °C were 28 ± 3 and 33 ± 3 mmol·s^{-1}·litre of cells^{-1} respectively.

Table 2. Permeability coefficients for the exchange of 3FG across human red-cell membranes from four donors

Permeability coefficients were measured using the 'over-determined' one-dimensional exchange analysis as described in the Materials and methods section. The permeability coefficients in the first two columns are divided into groups obtained on different days. Error estimates in single determinations were made using the S.D. in each rate constant (obtained from the analysis) and the S.D. of the Ht and cell-count determinations. Where two determinations of the permeability coefficient were made on a single sample, the value in the Table is the weighted mean and error estimates are the weighted S.D. values.

	$10^5 \times$ Permeability coefficient (P) ($cm \cdot s^{-1}$)							
Donor...	1		2		3		4	
Anomer...	β	α	β	α	β	α	β	α
	3.5±0.3*	4.1±0.3*	3.4±0.2*	4.5±0.3*	3.2±0.2*	4.1±0.3*	3.4±0.2*	4.0±0.3*
	2.7±0.2*	4.5±0.3*	4.1±0.3*	4.3±0.3*	3.3±0.2	4.5±0.3	3.6±0.2	4.9±0.3
	3.9±0.3*	3.5±0.2*	3.4±0.2*	4.5±0.3*				
	3.8±0.2	3.9±0.2	3.3±0.2	4.3±0.3				
	3.3±0.2	4.2±0.3	3.2±0.2	4.2±0.3				
	3.0±0.2	4.2±0.3	3.9±0.2	4.4±0.3				

* These results represent the weighted mean of two determinations of the permeability coefficient on the same sample.

Table 3. Ratio of the permeability coefficients (P^β/P^α) for the exchange of α- and β-3FG in red cells from four donors

Results are derived from the data in Table 2. The anomeric preference of 3FG exchange for each donor was calculated from the ratio of the permeability coefficients (eqn. 11) for the two anomers (Table 2).

	P^β/P^α			
	1	2	3	4
	0.86±0.10	0.76±0.07	0.79±0.07	0.86±0.08
	0.61±0.06	0.96±0.10	0.74±0.07	0.74±0.07
	1.10±0.10	0.75±0.07		
	0.96±0.08	0.76±0.07		
	0.79±0.07	0.76±0.06		
	0.72±0.07	0.88±0.07		
Weighted mean ±weighted S.D. (n)...	0.79±0.07 (6)	0.80±0.07 (6)	0.77±0.07 (2)	0.82±0.11 (2)

Comparison of P^β and P^α in red cells from four donors

The permeability coefficients for exchange of β- and α-3FG across the red-cell membrane were also determined in red cells from four donors at a single (total) 3FG concentration. Permeability coefficients were measured at 37 °C as described above, but in the presence of 8 mM-glucose rather than 10 mM-inosine. The concentrations of β- and α-3FG were ~ 9.0 mM and ~ 7.7 mM respectively. The results are shown in Table 2. The permeability coefficients for the two anomers, measured in each sample, were compared by using Student's t test (modified to the method of paired comparisons as described by Bailey [17]), thereby eliminating the effects of either possible intrinsic differences in the transmembrane exchange of 3FG in red cells from the four donors or small differences in sample preparation. In analysing the 16 *pairs* of permeability coefficients, P^β was found to be significantly smaller than P^α [at the 0.1% level, i.e. P (probability) value < 0.001].

The values of P^β/P^α calculated for each sample are shown in Table 3. The weighted mean value of 0.79 ± 0.07 (where the error denotes the weighted S.D. of the results for the 16 samples) was consistent with the anomeric preference calculated in the experiments above (in which a range of 3FG concentrations was used). The weighted mean value of P^β/P^α was also calculated for each individual (Table 3). The weighted mean anomeric preferences for each of the four individuals lie within 1 S.D. of each other.

Comparison with previous n.m.r. results

The values of P^β/P^α in Table 3 differed considerably from that calculated in a previous experiment in which n.m.r. *saturation transfer* was used to measure the relative rates of the transmembrane exchange of β- and α-3FG in red cells from a single individual [16]. Thus, in the present work, P^β/P^α was also determined in red cells from one donor (Donor 1 in Tables 2 and 3) using saturation transfer. Two experiments were performed in which samples were prepared as previously described [16] or as described in the Materials and methods section. P^β/P^α in the two experiments was 0.77 ± 0.13 and 0.67 ± 0.12 respectively. The first value was determined from two measurements (for each anomer) on a single sample; the uncertainty was calculated from the range of the mean value for each permeability coefficient. The second value was calculated similarly to the first, but using two samples; thus we calculated the weighted mean of the anomeric preference in each sample using the uncertainty in the anomeric preference for each sample as the weighting factor. Both these values were within 1 S.D. of the mean value of P^β/P^α determined for Donor 1 using the 'over-determined' one-dimensional exchange analysis (Table 3); therefore the measured value of P^β/P^α appeared to be independent of the n.m.r. spin-transfer method employed.

DISCUSSION

The determination of the anomeric preference of exchange via the human red-cell glucose transporter is important for the accurate analysis of the many kinetic experiments that have been carried out on the transport of glucose across the human red-cell membrane. In a ^{19}F-n.m.r. spectrum of 3FG, the two anomers are easily distinguishable, so exchange of the two anomers can be measured in the same sample under the same conditions and at the equilibrium concentrations of the anomers. Thus we carried out a study of the anomeric preference of 3FG exchange using

blood from different donors and over a range of substrate concentrations.

Anomeric preference of exchange via the red-cell glucose transporter

The binding of glucose to the red-cell glucose-transport protein probably involves a hydrogen bond between a number of glucose hydroxy oxygen atoms (including the C-1 hydroxy group) and hydrogen atoms on the protein [2]. Since the β-D- and α-D-glucose molecules differ in the orientation of the hydroxy group on C-1, it would not be surprising if the two anomers bound with different affinity to the protein. Studies of the relative strength of binding of various substrates to the carrier indicated that the β-anomer of glucose would bind with greater affinity than the α-anomer [2,6].

Direct measurements of the rates of exchange of the two anomers obviate the need to extrapolate relative strengths of binding to relative rates of exchange. However, the task of directly measuring the rates of exchange of the two glucose anomers across the red-cell membrane under physiological conditions is made difficult by the rapid rate of exchange at 37 °C.

In previous studies, Faust [7] observed that, at a glucose concentration of 300 mM, the β-anomer was transported approximately three times more quickly than the α-anomer, but Carruthers & Melchior [25] suggest (for a number of reasons) that these results may be subject to systematic errors. In their study of the anomeric preference of glucose exchange at a concentration of 100 mM, they reported that, in 22 experiments on the α-anomer and 18 experiments on the β-anomer, the mean half-time for equilibration under infinite-*cis* conditions is indistinguishable for the two anomers. However, as Lowe & Walmsley [3] pointed out, this conclusion is based on data with very high standard deviations. Fujii and co-workers [8], from a study carried out at 25 °C and at a glucose concentration of 17 mM, reported that the β-anomer is transported significantly, but not markedly (1.13 times), more quickly than the α-anomer. Appleman & Lienhard [9], in studies of the interconversion of the inward- and outward-facing conformers of the purified transporter reconstituted into membranes of red-cell lipids, observed that the α-anomer of glucose was 37% more effective than the β-anomer in facilitating the interconversion.

The fluorinated glucose derivative 3FG has previously been shown to be transported by, and to bind to, the human red-cell glucose transporter with a higher apparent affinity than glucose [26]. By using this compound in ^{19}F-n.m.r. magnetization-transfer experiments, we were not only able to measure exchange through the glucose-transport protein under equilibrium-exchange conditions at 37 °C, but, because the ^{19}F-n.m.r. resonances from the two anomers were well-separated (Fig. 1*a*), we could measure exchange of both anomers in the same sample [16]. Thus, in the present work, we determined the anomeric preference of 3FG exchange over a range of concentrations and found consistently that the α-anomer of 3FG was transported across the red-cell membrane with a smaller K_m (greater apparent affinity) than the β-anomer (Table 1). This conclusion was reached from the exchange-rate measurements after assuming that the maximal velocities of exchange of the two anomers were not significantly different [22]. The experiments were carried out with inosine present as the glycolytic substrate, to avoid the complication of non-fluorinated glucose competing with 3FG exchange.

In a study of the anomeric preference of 3FG exchange in red cells from four individuals at a single 3FG concentration (Tables 2 and 3), we observed that the α-anomer was transported across the membrane with greater apparent affinity than the β-anomer. This result is consistent with the results obtained using a range of 3FG concentrations and red cells from a single individual.

Steady-state rate parameter values

The absolute values of K^α and K^β were calculated from the substrate-concentration-dependence of the permeability coefficients for the two anomers. Recently, Gasbjerg & Brahm [27] determined the half-saturation constant for glucose efflux under self-exchange conditions at 20 °C to be 10 ± 1 mM. Lowe & Walmsley [28] found that the K_m for glucose influx under equilibrium-exchange conditions varied between 12 and 17 mM as the temperature was increased from 0 to 43 °C. Thus the values of K^β and K^α obtained in the present work (3FG exchange under equilibrium-exchange conditions) at 34 °C of 8.8 ± 1.3 mM and 6.0 ± 0.9 mM respectively, and at 37 °C of 7.0 ± 1.0 mM and 8.3 ± 1.3 mM respectively, are consistent with the previous finding [26] (made at 37 °C measuring net efflux) that 3FG is transported across the human red-cell membrane with an apparent affinity that is marginally higher than that for glucose transport. The values of $V_{max.}$ measured for 3FG exchange were, at 34 and 37 °C, 28 ± 3 and 33 ± 3 mmol·s^{-1}·litre of cells^{-1} respectively. Lowe & Walmsley [28] measured a $V_{max.}$ for glucose influx at 37 °C, under equilibrium-exchange conditions, of ~ 23 mmol·s^{-1}·litre^{-1}.

Comparison with previous n.m.r. results

In a previous paper [16] we first reported the use of ^{19}F-n.m.r. and 3FG for measuring exchange across the human red-cell membrane via the glucose-transport protein. In demonstrating the effectiveness of spin-transfer techniques for measuring 3FG exchange, we used saturation transfer to measure the first-order rate constants for the transmembrane exchange of the α- and β-anomers of 3FG and concluded that the β-anomer was transported significantly more quickly than the α-anomer. As the previous results [16] were obtained by using a different n.m.r. spin-transfer method (saturation transfer) from that used in the present work ('over-determined' one-dimensional exchange analysis), we also measured the anomeric preference of 3FG exchange in the present work using saturation transfer; the results were the same (within experimental error) as those obtained using the more recent spin-transfer analysis.

Although there is a discrepancy between the anomeric preference of the human red-cell glucose transporter reported previously [16] and that observed in the present study, we have established that the smaller dispersion between the intra- and extra-cellular resonances observed in the previous experiments (in which the spectra were acquired at a lower magnetic field) resulted in an underestimation of the permeability coefficients. Specifically, as the dispersion of the intra- and extra-cellular resonances of the α-anomer was less than for the β-anomer, the permeability coefficient for the α-anomer was *under*estimated relative to that for the β-anomer. We compared the permeability coefficients for the α- and β-anomers measured in the previous work using Student's *t* test and found that, after correction for the above effect, there was no basis for claiming a significant difference between the permeability coefficients ($P > 0.05$) given the size of the errors in the parameter estimates. The above-mentioned correction of the previously measured permeability coefficients resulted on average in a 26% increase in the permeability coefficient for the α-anomer and a 14% increase in the permeability coefficient for the β-anomer. The greater dispersion between the intra- and extra-cellular resonances and much better signal-to-noise ratio in the spectra that were obtained in the present work minimized the above effect in the saturation-transfer experiments and also facilitated the application of the 'over-determined' one-dimensional exchange analysis.

In the present work, the mean permeability coefficients measured at a total 3FG concentration of 10.2 mM and at 37 °C were $(7.3 \pm 0.5) \times 10^{-5}$ cm·s^{-1} and $(5.8 \pm 0.4) \times 10^{-5}$ cm·s^{-1} for α- and β-3FG respectively. Previously we measured the permeability coefficients for the α- and β-anomers of 3FG, at 37 °C and a total concentration of ~ 9.3 mM, of $(2.2 \pm 0.8) \times 10^{-5}$ cm·s^{-1} and $(4.1 \pm 0.7) \times 10^{-5}$ cm·s^{-1} respectively, where these values have been corrected for the 'spill-over' effect described above [16]. (Note that in the previous paper [16] the term '$\times 10^{-5}$' was incorrectly absent from the text.) Thus, even after correction for spillover, the permeability coefficients for both the α- and β-anomers of 3FG were measured to be significantly higher in the present work, compared with that measured previously.

Conclusions

In the present work we consistently observed that, at 37 °C and under equilibrium-exchange conditions, the α-anomer of 3FG was transported across the human red-cell membrane with greater apparent affinity than the β-anomer. The discrepancy between this observation of the *relative* rates of exchange of the two anomers of 3FG across the red-cell membrane and a previous result obtained in a less extensive study, in which the splitting of the ^{19}F-n.m.r. resonances of the intra- and extra-cellular 3FG populations was reported [16], appears to have arisen partly as a consequence of the lower dispersion of the intra- and extracellular resonances in the previous work. It is, however, impossible to rule out biological variations between the samples that were used as a further explanation for the difference between the results of the two sets of experiments.

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