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Glucose transport in human erythrocytes measured using ^{13}C NMR spin transfer

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We present the results of a new NMR-based procedure for measuring the fast transmembrane exchange of D-[1-^{13}C]glucose in human erythrocytes. The method relies on different rates of exchange between the α- and β-anomers of glucose inside and outside the cells; the rate outside the cells is greatly increased by the addition of mutarotase to the suspension. Theory is developed to describe nuclear-spin transfer in the present system and is used to analyse the data to yield estimates of transmembrane-exchange rate constants and their statistical uncertainties. For a total glucose concentration of 25.5 mmol/l at 40° C the first order efflux rate constants for the α- and β-anomers were $1.20 \pm 0.40\ s^{-1}$ and $0.71 \pm 0.30\ s^{-1}$, respectively.

Glucose transport; ^{13}C-NMR; Spin transfer; (Human erythrocyte)

1. INTRODUCTION

Glucose transport across the membranes of human erythrocytes is fast; the half-life in cells with glucose at the normal plasma levels ($\sim 5\ mmol \cdot l^{-1}$) is ~1 s [1]. Analysis of transport rates under various experimental conditions is necessary for an understanding of the mechanism of the transport process. Glucose transport in human erythrocytes, liver cells, and many other cell types is carrier-mediated. As a result of using techniques of molecular biology, the complete primary structure of the carrier protein has recently been determined [2]. Antibodies to the liver protein cross-react with the transporter of the red cell thus suggesting that the protein moiety is the same; although the oligosaccharide chain attached to the protein is known to be tissue specific [2]. The latter information together with the results of many other experiments has resulted in a situation where the understanding of the structure of the transport protein is well in advance of the understanding of its mechanism of action. This situation is a result of a general paucity of experimental techniques for studying fast transmembrane processes. Stopped-flow procedures have been used to obtain initial velocities of glucose flux, in the sub-second time domain [3,4]. The rates of unidirectional flux of the α- and β-anomers of glucose were estimated from measurements of the rate of change of cell volume [5,6]. The earlier work [5] indicated a three-fold difference in rate between the anomers, but no difference was found recently [6]. Radioactive tracer-exchange studies at 20°C have yielded valuable insights into the mechanism of transport [7]. Other studies using centrifugal washing of cells, after incubation with the labelled glucose at 0°C (to slow down the exchange rates), have also contributed mechanistic insights [8].

NMR spin-transfer procedures offer the ability to study fast chemical exchange events, non-invasively, in both non-catalysed and enzyme catalysed reactions [9,10]. Recently such an NMR method was used to measure the rapid transport of dimethyl methylphosphonate across the human red

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cell membrane at 37°C [11]. An extension of this spin-transfer procedure has recently enabled the measurement of the exchange rate, at 37°C, of $H^{13}CO_3^-$ in these cells [12]. We present here the new method applied to measuring the exchange rate of each anomer of ^{13}C-labelled glucose at chemical equilibrium in red cells at physiological temperatures.

2. THEORY

The ^{13}C NMR spectrum of D-[1-^{13}C]glucose in a red cell suspension (see section 4) has two peaks, one each for the α- and β-anomers. In addition the peaks are split into two unresolved components with the separation being smaller for the α-pair. In both cases the high frequency component is due to the intracellular glucose (see section 5). Because the intra- and extracellular peaks are not resolved, a direct saturation transfer analysis (e.g. as in [11]) cannot be used for glucose transport. However, we can adapt the method recently used to measure the rate of $H^{13}CO_3^-$ exchange [12].

Consider fig.1 which is the reaction scheme involving glucose exchange in a cell suspension. Transmembrane exchange is rapid (but 'slow' on the NMR time scale [12]) while spontaneous anomerization is slow (half-life > 10 min). If mutarotase, which does not enter the cells, is added to the suspension, the rate of anomerization can be made arbitrarily large, depending on the enzyme concentration. In a separate experiment the rate constant of mutarotase-catalysed exchange can be measured by saturation or inversion transfer NMR spectroscopy (e.g. [9,10]).

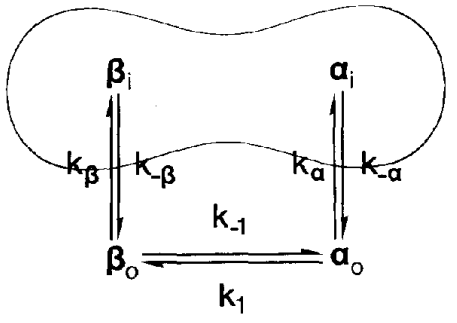

Fig.1. Scheme depicting the transmembrane exchange of α- and β-anomers of glucose across the human red cell membrane, and the enzyme-catalysed anomerization by extracellular mutarotase. α and β denote the respective anomers, and the subscripts, i and o, specify intra- and extracellular. The ks denote the respective apparent first-order rate constants for exchange.

The present theory is developed for the β-anomer but entirely analogous expressions apply to the α-anomer. The degree of saturation transfer (from α- to β; ζ_β) is defined as the normalized difference between the total signal intensity of the β resonance with and without saturation by selective radiofrequency irradiation of the sample at the α-resonance frequency: viz.,

$$\zeta_\beta = \frac{(\beta_o^E + \beta_i^E) - (\beta_o + \beta_i)}{(\beta_o^E + \beta_i^E)}, \tag{1}$$

where $(\beta_o^E + \beta_i^E)$ and $(\beta_o + \beta_i)$ are the total areas (intensities) of peaks in the absence and presence of α-saturation, respectively. For pure supernatant (i.e., no β_i) the degree of saturation transfer for our simple two-site system is [9],

$$\zeta_{\beta_o} = k_{-1}\, T_{\beta_o}^{SAT}, \tag{2}$$

where the subscript o denotes 'outside' the cells. $T_{\beta_o}^{SAT}$ is the longitudinal relaxation time of the β-anomer population whilst the α-anomer is saturated. Thus from $T_{\beta_o}^{SAT}$ and ζ_{β_o} the value of the exchange-rate constant, k_{-1}, is obtained. In human red cells there is no saturation transfer between anomers of glucose (see section 4) so $\zeta_{\beta_i} = 0$ and $T_{\beta_i}^{SAT} = T_{\beta_i}$. Therefore, using our previous theory [12] we obtain an expression for the rate of β-anomer efflux from the red cells:

$$k_{-\beta} = \frac{\left\{\dfrac{\beta_o^E \zeta}{(\beta_i^E + \beta_o^E)}\right\} - \zeta}{\left\{(\zeta - \zeta_o) T_{\beta_i}^{SAT} + \dfrac{\beta_i^E}{\beta_o^E}\,\zeta\, T_{\beta_o}^{SAT}\right\}} \tag{3}$$

where $\beta_i^E = H\theta V[\beta_i^E]$, (4)

and $\beta_o^E = (1 - H)V[\beta_o^E]$. (5)

H is the haematocrit, V is the sample volume in the NMR transmitter/receiver coils (it is included here for rigour, but in fact it cancels-out of eqn 3), θ is the fraction of cell volume that is accessible to glucose, and the square brackets denote molar concentration.

3. MATERIALS AND METHODS

3.1. *Materials*

Cytochalasin B, dithiothreitol (DTT) and mutarotase (aldose-1-epimerase, EC 5.1.3.3; from porcine kidney, crystalline in 3.2 mol/l $(NH_4)_2SO_4$, 5800 IU/mg, 5 mg/ml) were obtained from Sigma, St. Louis, MO, USA. ^2H_2O (99.75%) was from the Australian Institute of Nuclear Science and Engineering, Lucas Heights, NSW, Australia. D-[1-^{13}C]Glucose was from ICN, Cambridge, MA, USA. All other reagents were of AR grade.

3.2. *Erythrocytes*

Freshly drawn venous blood (from P.W.K.) was centrifugally washed once in 4 vols of cold (4°C) isotonic saline [12]. After aspirating the buffy coat the red cells were suspended in 4 vols of cold phosphate-buffered saline (PBS; 20 mmol/l Na_2HPO_4, 147 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1 mmol/l DTT) in 1:4 $^2H_2O/^1H_2O$, adjusted to pH 7.4 with NaOH using a glass electrode. The cell suspension was bubbled with CO for 10 min and then washed twice in PBS. Then 6 ml of PBS containing 60 mg of D-[1-^{13}C]glucose was added to 10 ml of the cell pellet; this suspension was respun and 4 ml of the packed cells were set aside for NMR analysis. The remaining sample was resuspended in the supernatant and then 0.4 ml of the mutarotase solution (vide infra) was added. After centrifugation 3 ml of the supernatant was set aside, and two samples of cells of haematocrit (H) = 0.64 were composed from the cell pellet and supernatant. Utilization of glucose by the cells was followed by ^{13}C NMR: after 1.5 h >90% remained.

Cytochalasin B, a glucose transport inhibitor [13], was added as 5 μl of suspension (1.0 mg in 25 μl of ethanol) to 3 ml of cell sample.

3.3. *Mutarotase*

0.4 ml of the enzyme suspension was diluted with 1.5 ml PBS in a Centricon microconcentrator (10 kDa cutoff; Amicon Corp., Danvers, MA, USA). The microconcentrator was centrifuged at 5000 × g for 100 min at 15°C. Two further washes of the enzyme retentate were carried out with 2.0 ml PBS. The final volume of the enzyme solution was adjusted to 0.4 ml with PBS.

3.4. *NMR*

3-ml samples were placed in 10 mm (outer diameter) tubes. The ^2H_2O in the buffer was for field/frequency locking. ^{13}C NMR spectra were acquired at 100.62 MHz in the Fourier mode using a Varian XL-400 spectrometer. Selective irradiation employed the DANTE pulse sequence [14]; pulse widths and delays were as described [10], and in the T_1^{SAT} measurements the DANTE pulse train was applied for 30 s (>5T_1). To minimise cell sedimentation the samples were not spun, and the probe was thermostatted to 37°C. Broad band proton decoupling led to an additional 3°C temperature rise in the sample.

3.5. *Data analysis*

T_1^{SAT} estimates were made using the standard Varian software which involves a three parameter fit; we used 10 data pairs with duplicate zero-time points. Calculations of membrane exchange-rate constants were made using eqn 3 programmed in BASIC in a Tektronix 4052 graphics computer. Standard deviations of $k_{-\beta}$ and $k_{-\alpha}$ were computer calculated using the formula for the variance of a function of several variables [15]. The partial deviation of $k_{-\beta}$ with respect to ζ, ζ_o, $T_{\beta i}^{SAT}$, $T_{\beta o}^{SAT}$, θ and H were obtained from eqn 3 and programmed in BASIC; the input to the program included the standard derivation of each of these experimental variables.

4. RESULTS

Fig.2A is the ^{13}C NMR spectrum of supernatant removed from red cells in PBS-mutarotase (0.2 mg/ml). Stability of mutarotase was maintained by DTT and EDTA in the PBS. The natural line-width at half peak height for each anomer was 1.6 Hz and the signal-to-noise ratio for several such solutions was in excess of 100:1. The ratio of peak areas (intensities) gave the equilibrium constant for the anomerization reaction as 1.69, or relative concentrations of 62.3% β- and 37.7% α-anomer. Saturation of the β-anomer led to the result in fig.2B: the degree of saturation transfer ($\zeta_{\alpha o}$; eqn 2) was 0.813. This value, coupled with the measured T_α^{SAT} = 0.41 ± 0.03 s, gave k_1 = 1.98 s^{-1}.

Fig.3 shows expanded-scale plots of the α- and β-anomer peaks of D-[1-^{13}C]glucose from a

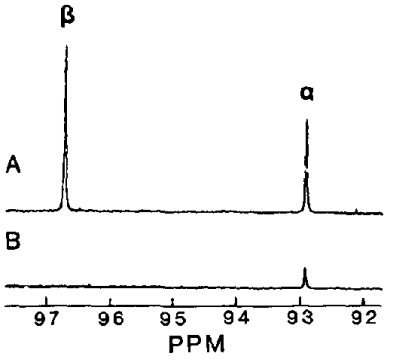

Fig.2. ^{13}C NMR saturation transfer from the β- to the α-anomer of D-[1-^{13}C]glucose in the supernatant from a red cell suspension containing mutarotase. The glucose concentration was 25.5 mmol/l; details of sample preparation given in section 3. (A) Control spectrum with selective irradiation to the low-frequency side of the α-resonance. (B) Selective irradiation at the frequency of the β-resonance. NMR parameters: spectral width, 1000 Hz; 8000 data points per transient; line broadening factor, 1 Hz; and inter-transient delay, 20 s.

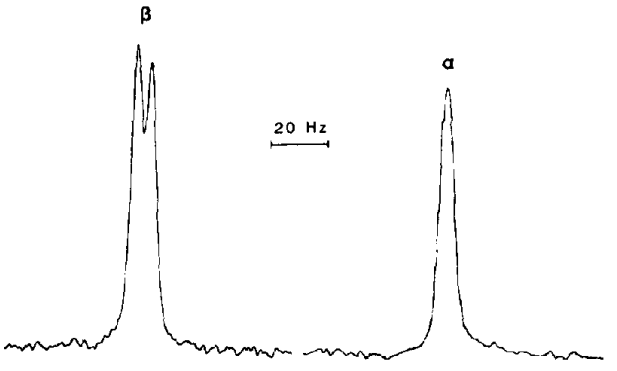

Fig.3. ^{13}C NMR resonances of D-[1-^{13}C]glucose in a suspension of human red cells. The glucose concentration was 25.5 mmol/l and the haematocrit was 0.64. The sample, which contained mutarotase in the extracellular space, was prepared as described in section 3. NMR parameters were as for fig.2.

suspension of red cells. The most notable feature is the partial resolution of the β-resonance into two components, and in the present case only broadening of the α-resonance (in other samples a 'split' α-peak was also evident); the line-widths at half peak height were 9.3 Hz for the β and 5.2 Hz for the α peak; the frequency difference of the β-peaks was 5.0 Hz.

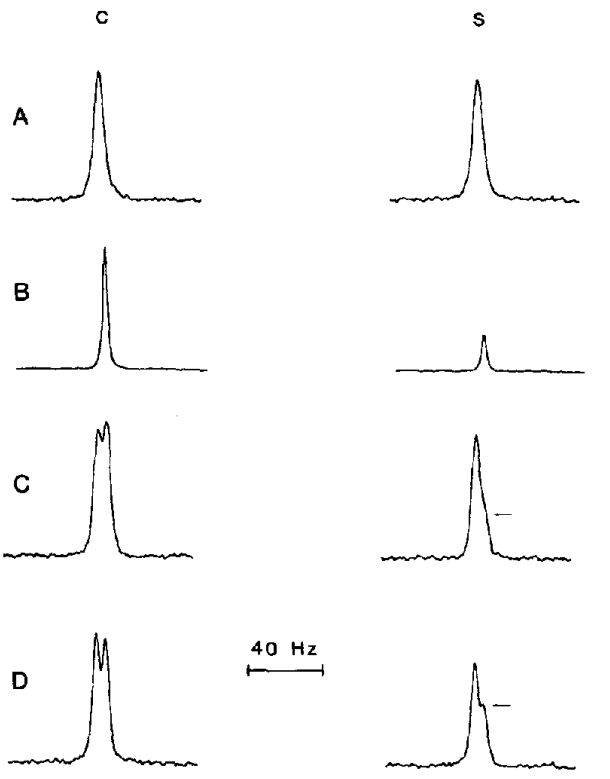

Fig.4. ^{13}C NMR saturation transfer from the α- to the β-anomer of glucose in: packed red cells (A); cell-supernatant (B); cells treated with cytochalasin B (C); and without the inhibitor (D). The left-hand column (c) contains the control spectra and the right-hand column (s) the saturation transfer spectra. Sample preparation was as described in section 3. NMR parameters were as for fig.2. The arrows in C and D indicate the extracellular component of the β-resonance.

Fig.4 contains four pairs of spectra showing only the resonance of the β-anomer of glucose. The left-hand column (denoted c for 'control') was obtained with selective irradiation of the sample, at a frequency displaced from the β-anomer frequency by a value equal to the $\alpha - \beta$ difference. The right-hand column of spectra was obtained with saturation of the α-anomer. Integration of the peaks in fig.4A revealed them to be the same. Thus no saturation transfer occurs between the anomers inside the cells; in other words $f_{\beta_i} = 0$. For supernatant (fig.4B) the degree of saturation transfer, f_{β_o}, was 0.673. In a cell suspension of haematocrit 0.64 (fig.4C), in the presence of the glucose transport inhibitor cytochalasin B, the value of f_β was 0.227.

The low frequency component of the glucose peak shows evidence of saturation transfer and is reduced to a shoulder (marked by an arrow). In the absence of inhibitor (fig.4D), glucose exchange between intra- and extracellular compartments results in transfer of saturation to the intracellular component of the peak; there is also a concomitant decrease in saturation of the extracellular component (marked by an arrow) relative to that seen in fig.4C. The total degree of saturation transfer, in the absence of inhibitor was 0.350.

The T_1^{SAT} values for each anomer inside and outside the cells were: $T_{i\beta}^{SAT} = T_{i\beta} = 0.98 \pm 0.02$ s; $T_{i\alpha}^{SAT} = T_{i\alpha} = 0.93 \pm 0.03$ s; $T_{o\beta}^{SAT} = 0.59 \pm 0.01$ s; and $T_{o\alpha}^{SAT} = 0.41 \pm 0.03$ s. We took θ to be 0.7 [11] $\pm$ 0.02, and assumed the standard deviation of an haematocrit estimate to be 0.005. By combining these data, with those obtained from fig.4A,B and D, into eqn 3 we obtained $k_{-\beta} = 0.86 \pm 0.40$ s^{-1} and $k_{-\alpha} = 0.94 \pm 0.30$ s^{-1}. Furthermore, the rate constants from the means of three separate experiments, conducted on the same samples, were 0.71 ± 0.30 s^{-1} and 1.20 ± 0.40 s^{-1}.

5. DISCUSSION

The α/β anomer ratio of glucose in PBS, either in the presence or absence of mutarotase, or in cells, was the expected value [16], 37.7%/62.3%. The lack of saturation transfer between the α- and β-resonances in packed cells was consistent with the observation of Carruthers and Melchior [6] that there was no detectable mutarotase activity in erythrocytes at 0.6°C.

The physical basis of the split β- and broad α-resonances is not yet known. However, it is not solely due to a difference in magnetic susceptibility since the extent of splitting is different for each anomer. Fig.4C shows that saturation from the α-anomer is transferred to the low-frequency peak of the β-anomer, thus assigning the latter to the extracellular component. The complementary saturation transfer from β to α (not shown) led also to suppression of the low-frequency side, and a narrowing, of the α-resonance. Thus, the fact that saturation transfer occurs to the low-frequency side of both resonances implies that mutarotase-catalysed exchange, which could in theory lead to intermediate-to-fast exchange and hence a moving together of the resonances, is also not the explanation for the splitting. Therefore, we tentatively postulate that the effect is due to a small amount of glucose binding inside the cell; the resonance frequencies of the free and bound forms are presumably different and the chemical exchange presumably is rapid (on the NMR time scale). The amount of bound glucose in the cell is small since the concentration of glucose, estimated from the amount inside and the cell-water volume, is equal to that outside the cells; this appears to be true for glucose concentrations less than ~30 mmol/l [17].

Further evidence that the low-frequency component of the α- and β-peaks were due to extracellular glucose was obtained by adding the 'paramagnetic broadening' agent, $MnCl_2$, to a cell suspension that was treated with cytochalasin B. The low-frequency component of the β-peak was suppressed, and narrowing of the α-peak occurred, presumably due to the elimination of the unresolved extracellular component.

The present analysis yielded estimates of the apparent first-order rate constants for transmembrane exchange of α- and β-anomers at equilibrium. The values of the two efflux rate constants differed by less than the sum of their standard deviations, thus implying that, under the present conditions of buffer and substrate concentration there is no differential transport rate of the anomers. This is consistent with other work [6]; but more detailed kinetic analysis is required with our system.

In conclusion, we have developed a new procedure, based on NMR spin-transfer theory, for studying the rapid exchange of ^{13}C-labelled glucose in human red cells at physiological temperatures. The apparent first-order rate constants measured for the two glucose anomers were in accord with those obtained with technically less direct procedures; the results imply a half-life in human red cells of glucose, at 25.5 mmol/l, of ~1 s. Extension of the experiments to different glucose concentrations, different temperatures, and protein modification, may yield further insight into the mechanism of operation of this important transport protein as it functions in the red cell and other tissues, in situ.

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