Biophysical Chemistry, 32 (1988) 89–95
Elsevier

BPC 01315

Mutarotase equilibrium exchange kinetics studied by ^{13}C-NMR

Philip W. Kuchel, Brian T. Bulliman and Bogdan E. Chapman

Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia

Received 29 March 1988
Accepted 25 July 1988

Mutarotase; Aldose-1-epimerase; Glucose anomerization; two-dimensional NMR spin-exchange spectroscopy; (Porcine kidney)

The rates of exchange between the α- and β-anomers of D-[1-^{13}C]glucose, at equilibrium catalyzed by porcine kidney mutarotase (EC 5.1.3.3), were measured using ^{13}C-NMR spin-transfer procedures. This entailed inversion-transfer and saturation-transfer experiments, and two-dimensional exchange spectroscopy (2D EXSY). The concentration and temperature dependences of the fluxes were studied; equilibrium exchange Michaelis constants, and the activation energy of the catalyzed reaction were thereby measured.

1. Introduction

The enzyme mutarotase (aldose-1-epimerase; EC 5.1.3.3) catalyzes the interconversion (anomerization) of the α- and β-anomers of a range of aldoses [1]. It is widely distributed in biology, and in mammals the highest activities are found in the kidney [2]. Multiple forms of the enzyme (isoenzymes) exist in mammals [3], and the bovine kidney enzyme(s) have their highest maximal velocities with several aldo-pentoses, while amongst the aldo-hexoses D-galactose and D-glucose are the 'preferred' substrates [4].

Assay methods for the enzyme in the past have involved; (a) measurement of the catalyzed rate of mutarotation of the pure α-anomer by semi-automated polarimetry (e.g., see refs. 1, 5 and 6), and (b) the fast generation of the α-anomer from sucrose by β-fructofuranosidase (invertase) [7] followed by β-D-glucose oxidase catalyzed oxidation of the β-anomer with production of hydrogen peroxide, the latter being detected by reaction with a redox chromogen [7]. The large chemical shift difference between the resonances of the anomeric carbons of D-[1-^{13}C]glucose in the ^{13}C-NMR spectrum has been known for many years [2,8]; yet the present work appears to be the first to employ NMR spin-exchange procedures to measure exchange flux in the catalyzed system at equilibrium.

We have recently developed a ^{13}C-NMR procedure to measure the rapid exchange of D-[1-^{13}C]glucose across the human erythrocyte membrane [9]. For this procedure a kinetic characterization of mutarotase, under the pH and buffer conditions used, is valuable for the purpose of experiment design. Thus, we describe here the measurement of (a) the Michaelis constant at pH 7.4 in phosphate-buffered saline, (b) the overall equilibrium constant of the reaction and (c) the activation energy of the reaction. Both of the latter values agree with earlier estimates, yet the activation energy derived from the equilibrium-exchange rate must be interpreted in terms of a particular reaction scheme; this matter is addressed in section 4.

From a methodological perspective the present work entailed a comparison of several NMR spin-transfer procedures used to measure the flux of solutes between two chemically distinct sites. Accordingly, we compared fluxes measured with saturation-transfer [10], inversion-transfer [11] and

Correspondence address: P.W. Kuchel, Dept of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia.

two-dimensional exchange spectroscopy (2D EXSY) [12]; excellent agreement between estimates was in general obtained.

2. Materials and methods

2.1. Materials

D-[1-^{13}C]Glucose was obtained from ICN (Cambridge, MA, U.S.A.). Dithiothreitol and mutarotase (from porcine kidney, crystalline in 3.2 mol/l ammonium sulfate, 5800 I.U./mg, 5 mg/ml) were obtained from Sigma (St. Louis, MO, U.S.A.). ^2H_2O (99.75%) was from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, N.S.W., Australia). All other reagents were of AR grade.

2.2. Mutarotase preparation

The enzyme suspension (0.4 ml) was added to 1.5 ml phosphate-buffered saline (20 mmol/l Na_2HPO_4, 127 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1 mmol/l dithiothreitol, in 1 : 4 $^2H_2O/^1H_2O$) in a Centricon microconcentrator (10 kDa cut-off; Amicon Corp., Danvers, MA, U.S.A.). The microconcentrator was centrifuged at $5000 \times g$ for 60 min at 15° C. Three further washes of the enzyme retentate were carried out with 2 ml phosphate-buffered saline. The ^2H_2O in the phosphate-buffered saline was for field-frequency locking in the NMR spectrometer.

2.3. ^{13}C-NMR

^{13}C-NMR spectra were recorded at 100.62 MHz in the Fourier mode using a Varian XL400 spectrometer, with 3-ml samples in 10-mm NMR tubes. Saturation-transfer measurements were carried out using a DANTE pulse sequence [13]. Typically 8 or 16 transients were averaged into 8K data points with a spectral width of 1200 Hz. The DANTE pulse width was 2 μs (the 90° pulse was 28 μs) and the delay between pulses was 333 μs, giving excitation side bands spaced 3000 Hz apart.

Broad-band ^{1}H decoupling was used in all experiments, consequently it was necessary to measure directly the sample temperature; this was achieved using the procedure of Bubb et al. [14].

The δ-ordered inversion-transfer pulse sequence was used in the inversion-transfer experiments [15].

Two-dimensional phase-sensitive EXSY spectra were obtained using the hypercomplex method of States et al. [16]. Eight transients were added into 1024 memory points using 128 t_1 increments and zero filling to 1024 points in the f_1 domain.

Longitudinal relaxation times (T_1) of spin populations were measured by non-linear regression onto the data from the inversion recovery pulse sequence [17].

2.4. Numerical procedures

The two-site inversion-transfer data consisted of two sets of complementary experiments [18]; viz., initial inversion of the β-anomer resonance and transfer via the enzyme of this inversion to the α-anomer, and then the opposite experiment, thus yielding four magnetization time courses. The relevant Bloch-McConnell equations describing the exchanging system [19] were integrated numerically using a third-order semi-implicit Runge-Kutta algorithm [20]. The sum of squares of differences (residuals) between the simulation and the data, for given times in a time course, was added to those of the corresponding times in the other three time courses, and the whole was minimized using a non-linear regression program [21]. The 'best' estimates of the rate constants together with their variances resulted from this analysis.

The matrix manipulation necessary for analysis of the 2D EXSY spectra used standard eigenvalue/eigenvector algorithms [12].

3. Results

3.1. 2D NMR kinetic analysis

The 'fully relaxed' ^{13}C-NMR spectrum of a range of D-[1-^{13}C]glucose concentrations (9.2–115 mmol/l; 10 estimates at evenly spaced concentrations) gave β-/α-anomer mole ratios, which were independent of concentration, of 1.68 ± 0.06. This

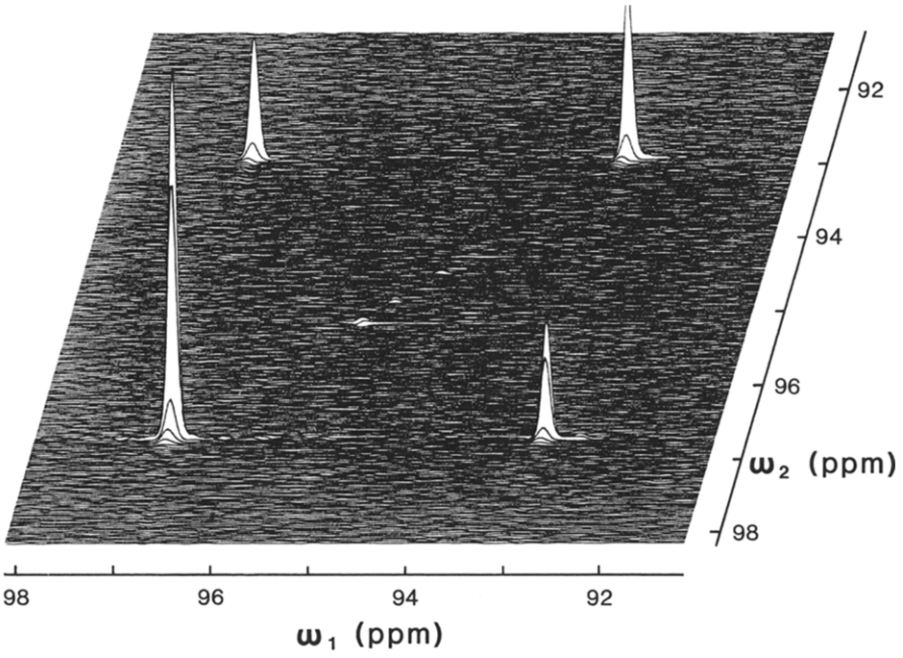

Fig. 1. ^{13}C-NMR 2D EXSY spectrum of D-[1-^{13}C]glucose (18.4 mmol/l) with mutarotase (0.8 mg/ml) at 37°C. The buffer was phosphate-buffered saline, pH 7.4, and sample preparation was according to section 2. The relative peak volumes are given in eq. 4.

ratio (K_{eq}) was not perceptibly altered in the presence of mutarotase at a concentration of around 1 mg/ml.

A 2D EXSY spectrum of the anomerizing system, with 18.4 mmol/l glucose, is shown in fig. 1. The spectrum was analyzed quantitatively to determine the first-order rate constants characterizing the flux between the two anomers [12]. In accordance with the theory, the 2D spectrum is viewed as the solution of the Bloch-McConnell equations that describe relaxation and exchange of magnetization in the chemical system,

$$\alpha \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \beta. \tag{1}$$

Thus,

$$\boldsymbol{M} = \exp(\boldsymbol{R}t_m)\boldsymbol{M}_0, \tag{2}$$

where $\boldsymbol{M}$ is the matrix comprising the peak volumes of the 2D EXSY spectrum, $\boldsymbol{R}$ the matrix of exchange and relaxation-rate constants relevant to the system under study, and t_m the, so-called, mixing time in the experiment [12]. $\boldsymbol{M}_0$ is a diagonal matrix representing the 2D EXSY spectrum acquired with $t_m = 0$. To avoid the need for a second time-consuming 2D EXSY experiment, we replace $\boldsymbol{M}_0$ with $\zeta\boldsymbol{S}_0$ where $\boldsymbol{S}_0$ is the diagonal matrix of the mole ratios of the fully relaxed 1D spectrum (discussed above), and ζ a scalar of unspecified value. The analysis, using $\zeta\boldsymbol{S}_0$, gives an $\boldsymbol{R}$ matrix whose off-diagonal elements are the exchange rate constants.

$$\boldsymbol{R} = \begin{bmatrix} \frac{1}{T_{1,\alpha}} + k_1 - \ln\zeta & -k_{-1} \\ -k_1 & \frac{1}{T_{1,\beta}} + k_{-1} - \ln\zeta \end{bmatrix} \tag{3}$$

From fig. 1 we obtained

$$\boldsymbol{M} = \begin{bmatrix} 0.429 & 0.149 \\ 0.149 & 0.210 \end{bmatrix} \tag{4}$$

where the EXSY peak volumes have been reflected about the last row to give the standard mathematical matrix orientation.

By using the back transformation analysis, with $t_m = 1$ s, we obtained

$$\boldsymbol{R} = \begin{bmatrix} 0.349 & -0.385 \\ -0.648 & 0.709 \end{bmatrix} \quad (5)$$

from which $k_1 = 0.648$ and $k_{-1} = 0.385$ s^{-1}.

3.2. Inversion-transfer kinetic analysis

Selective inversion of one or other of the anomer peaks in the NMR spectrum and then monitoring the return to equilibrium intensity of the peaks is another procedure whereby the equilibrium-exchange rate constants, k_1 and k_{-1}, can be determined. Fig. 2A shows the recovery of the inverted β-anomer resonance as a function of time, for a series of four glucose concentrations; the time course for a fifth concentration, 115 mmol/l, has been omitted from this figure for the sake of clarity. Fig. 2B shows the time dependence of the non-inverted α-anomer peak. Note that the

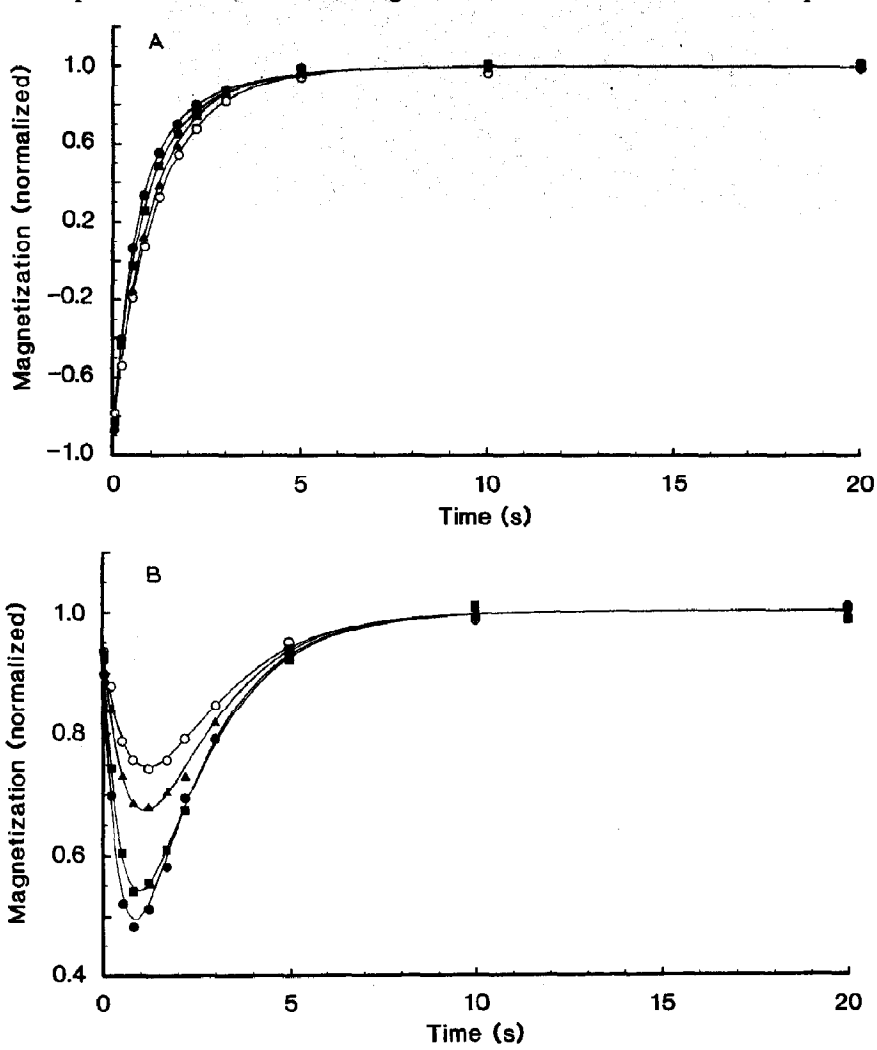

Fig. 2. ^{13}C-NMR magnetization-inversion transfer in the mutarotase-catalyzed exchange of the α- and β-anomers of D-[1-^{13}C]glucose at 37 °C. The sample preparation was as for fig. 1, and NMR procedures are described in section 2. Glucose concentrations were (mmol/l): (○) 69.0, (▲) 46.0, (■) 18.4, (●) 9.2. (A) Normalized (with respect to the equilibrium signal) β-anomer resonance intensity as a function of time after its selective inversion. (B) Normalized α-anomer resonance intensities, as a function of time, during the experiment described in A above. The solid lines are from non-linear regression analysis (see section 2).

Table 1

Glucose anomerization rate constants [a] for the mutarotase-catalyzed reaction estimated using inversion-transfer ^{13}C-NMR

Total glucose concentration (mmol/l)	k_1 (s^{-1})	k_{-1} (s^{-1})
9.2	0.851 ± 0.028 [b]	0.503 ± 0.016
18.4	0.687 ± 0.011	0.406 ± 0.007
46.0	0.374 ± 0.011	0.237 ± 0.007
69.0	0.278 ± 0.007	0.168 ± 0.005
119.0	0.156 ± 0.007	0.086 ± 0.007

[a] Estimated by non-linear regression analysis (section 2).
[b] Standard deviation.

peak intensity declines to a minimum at approx. 1 s and then relaxes back to equilibrium which is attained in approx. 10 s. The complementary experiments wherein the α-anomer resonance was inverted were also performed and the data from all four time courses (for a given glucose concentration) were analyzed simultaneously as described in section 2. The regression analysis involved 'floating' the following eight parameters: k_1, k_{-1}, the equilibrium magnetizations (signal) of α- and β-anomers, the T_1 values for each anomer, and the two zero-time magnetizations. Table 1 contains the estimates of the k parameters.

Multiplying the rate constants by their corresponding anomer concentration (obtained using the value of the equilibrium constant) gives the unidirectional flux, via the enzyme, from α- to β-anomer and vice versa. A graph of the reciprocal of the flux vs. the reciprocal of the free anomer concentration (viz., a Lineweaver-Burk plot) was linear for both cases. Regression of a line (weighted according to the variances of the k parameters [22]) onto the data yielded estimates of the Michaelis constants, under equilibrium exchange (ee) conditions which were $K^{ee}_{m,\alpha} = 6.1 \pm 1.4$ mmol/l and $K^{ee}_{m,\beta} = 7.1 \pm 2.6$ mmol/l.

Also, from the inversion-transfer data, we observed that there was no systematic dependence of T_1 values for the five glucose concentrations given in table 1. The mean values were $T_{1,\alpha} = 1.70 \pm 0.02$ s and $T_{1,\beta} = 1.76 \pm 0.02$ s.

3.3. Temperature dependence

Saturation transfer was used to measure the exchange-rate constants for two solutions of D-[1-^{13}C]glucose (9.2 and 43.2 mmol/l) with a fixed concentration of mutarotase (0.8 mg/ml) over the temperature range 8.9–34.2° C. The data were transformed to give an Arrhenius plot (fig. 3), and lines regressed onto the data had slopes, which when divided by the universal gas constant, gave estimates of the activation energy of the reaction. The estimates were 52.9 ± 5.0 and 48.0 ± 5.0 kJ/mol for the low and high glucose concentrations, respectively.

4. Discussion

The close similarity of the exchange-rate constants derived from the data in figs. 1 and 2 confirms the practical and theoretical equivalence of the two NMR experiments; this has also been shown recently for the non-enzymatic hydration of acetaldehyde [12].

Inversion of the anomeric hydroxyl group from an equatorial (β) to an axial (α) orientation in glucose is associated with a uniform increase in shielding of the glucose carbons (see fig. 1) and a decrease in shielding of the appended protons (evident in the ^{1}H-NMR spectra [23]) [2]. The similarity of $T_{1,\alpha}$ and $T_{1,\beta}$ values is consistent with dipole-dipole nuclear relaxation via the (non-exchanging) anomeric proton.

The simplest scheme for the mutarotase (E) reaction is,

$$\mathrm{E} + \alpha \underset{k_b}{\overset{k_a}{\rightleftarrows}} \mathrm{Ex} \underset{k_d}{\overset{k_c}{\rightleftarrows}} \mathrm{E} + \beta \qquad (6)$$

where the k terms are unitary rate constants, and α and β denote the respective anomers of glucose. The forward and reverse NMR tracer-exchange rates ($\nu_{\alpha\to\beta}$ and $\nu_{\beta\to\alpha}$, respectively) are interpreted in terms of the mechanism in eq. 6 as [19,24];

$$\nu_{\alpha\to\beta} = \frac{k_a k_c [\mathrm{E}]_0 [\alpha]_e}{k_b + k_c + k_a [\alpha]_e + k_d [\beta]_e} \overset{\text{def}}{=} k_1 [\alpha]_e, \qquad (7)$$

$$\nu_{\beta\to\alpha} = \frac{k_b k_d [\mathrm{E}]_0 [\beta]_e}{\text{denominator of eq. 7}} \overset{\text{def}}{=} k_{-1} [\beta]_e, \qquad (8)$$

where $[\mathrm{E}]_0$ is the total enzyme concentration, the

subscript e denotes equilibrium, and def means 'is defined as'; the latter implies that k_1 is the overall (apparent) exchange rate constant. From eqs. 6 and 7 the expressions for the equilibrium exchange Michaelis constants are,

$$K^{ee}_{m,\alpha} = \frac{k_b}{k_a}, \tag{9}$$

$$K^{ee}_{m,\beta} = \frac{k_c}{k_d}. \tag{10}$$

The maximal velocities of tracer exchange in each direction are, of course, equal:

$$V^{ee}_{max,forward} = V^{ee}_{max,reverse} = \frac{k_b k_c [E]_0}{(k_b + k_c)}. \tag{11}$$

Because the precise molar concentration of active enzyme was unknown in the experiments it was not possible to specify completely values for all the unitary rate constants. In principle, by studying the temperature dependence of the exchange rates, it is possible to determine these values [24]. However, in the present case, the analysis (not shown) did not yield meaningful results because the set of transformed data did not possess sufficient 'character' to allow unambiguous regression of a biexponential function onto them as required by the theory [24]. Nevertheless, it is possible to comment on the Michaelis constants: the ratio of these constants is given by

$$\frac{K^{ee}_{m,\alpha}}{K^{ee}_{m,\beta}} = \frac{k_b k_d}{k_a k_c} = \frac{1}{K_{eq}}. \tag{12}$$

From section 3.1 we note that $K_{eq} = 1.68$, yet the experimental ratio of K_m values is 1 : 1.16; this, in view of the variances of the data, is nevertheless consistent with the proposed reaction scheme given in eq. 6.

The spontaneous mutarotation of glucose, first reported in 1846 [25], is thought to occur via general acid-base catalysis, and is catalyzed efficiently by free amino acids such as histidine [26]. However, the reaction rate in single-component solutions is pH-independent over the range pH 3.2–6.0 [5]. On the other hand, the enzymatic reaction is pH-dependent and a histidyl has therefore been implicated in the active site [27]; yet

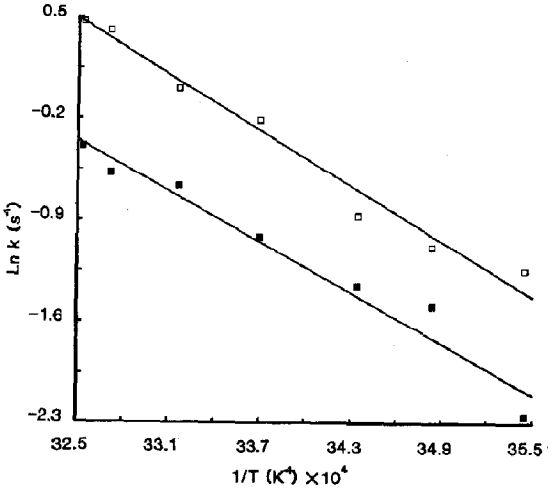

Fig. 3. Arrhenius plot showing the temperature dependence of the rate constant of mutarotase-catalyzed flux from the α- to β-anomer of D-[1-^{13}C]glucose, at equilibrium, measured with ^{13}C-NMR. Glucose concentrations were (□) 9.2 and (■) 43.2 mmol/l, and that of mutarotase was 0.79 mg/ml. The rate constants were obtained by using the saturation-transfer procedure with selective saturation of spin populations by the DANTE pulse sequence (section 2). The solid lines are unweighted regression lines with slopes corresponding to the activation energies (□) 52.9 and (■) 48.0 kJ/mol, respectively.

results, obtained with photo-oxidation reactions, on mutarotase [28] and the photo-affinity label phloretinyl-3′-benzylazide suggest involvement of a tryptophan residue in the active site [29]. The reported, initial velocity $K_{m,\alpha}$ value for the bovine kidney enzyme is 25 mmol/l at pH 7.4 and 25 °C [4], and for the porcine kidney enzyme it is 45 mmol/l at pH 6 [30]. Both values are much larger than we obtained for the equilibrium exchange values, but this can be explained by considering the expressions;

$$K_{m,\alpha} = \frac{k_b + k_c}{k_a}, \tag{13}$$

$$K_{m,\beta} = \frac{k_b + k_c}{k_d}, \tag{14}$$

from which it is clear that $K_{m,\alpha} > K^{ee}_{m,\alpha}$ because $k_c > 0$; and similarly $K^{ee}_{m,\beta} < K_{m,\beta}$. The activation energy of the mutarotase reaction, with 43.2 mmol/l D-[1-^{13}C]glucose, was calculated from fig. 3 to be 48.0 kJ/mol. The linearity of the plot

suggests that a single rate constant dominates the reaction rate; this is most probably the rate constant that describes dissociation of the enzyme-substrate complex. The apparently slightly higher value of the slope of the Arrhenius plot for the non-saturating glucose concentration (9.2 mmol/l) is not significantly different.

Another question relates to the equivalence of previously reported values for the activation energy (46 kJ/mol [4,28]), as determined from initial velocity estimates, and the present results obtained on the system at chemical equilibrium. The results can be shown to be equivalent as follows: let $k_b = \zeta k_c$, where ζ is an unknown scalar, then from eq. 11;

$$V_{max}^{ee} = \frac{\zeta k_c [E]_0}{(1+\zeta)}. \quad (15)$$

Hence, a plot of $\ln V_{max}^{ee}$ vs. $1/T$ will be a straight line with a slope determined by the activation energy relevant to k_c. Therefore, this slope is equal to that derived from the non-equilibrium exchange V_{max}, which is defined as $k_c[E]_0$.

In conclusion, we have defined the equilibrium exchange Michaelis constants of mutarotase for each anomer of glucose at pH 7.4. The activation energy of the reaction determined from equilibrium exchange experiments is theoretically and experimentally equal to that obtained from initial velocity studies. ^{13}C-NMR spin-transfer experiments appear to be a valuable adjunct to previously used procedures such as polarimetry, and stereo-specific enzymes with spectrophotometry, for studying the kinetics of mutarotase.

Acknowledgements

The work was supported by a grant from the Australian NH&MRC. Mr. W.G. Lowe is thanked for expert technical assistance.

References

1 J.M. Bailey, P.H. Fishman, J.W. Kusiak, S. Mulhern and P.G. Pentchev, Methods Enzymol. 44 (1975) 471.
2 R. Bentley, Annu. Rev. Biochem. 41 (1972) 953.
3 Y. Toyoda, I. Miwa and J. Okuda, J. Biochem. 94 (1983) 421.
4 J.M. Bailey, P.H. Fishman and P.G. Pentchev, J. Biol. Chem. 244 (1969) 781.
5 G.B. Levy and E.S. Cook, Biochem. J. 57 (1954) 50.
6 B. Andersen and F. Gronlund, Acta Chem. Scand. A, 33 (1979) 275.
7 M.K. Weibel, Anal. Biochem. 70 (1976) 489.
8 L.D. Hall and L.F. Johnson, Chem. Commun. (1969) 509.
9 P.W. Kuchel, B.E. Chapman and J.R. Potts, FEBS Lett. 219 (1987) 5.
10 S. Forsén and R.A. Hoffman, J. Chem. Phys. 39 (1963) 2892.
11 T.R. Brown, Phil. Trans. Roy. Soc. Lond. B, 289 (1980) 441.
12 P.W. Kuchel, B.T. Bulliman, B.E. Chapman and G.L. Mendz, J. Magn. Reson. 76 (1988) 136.
13 G.A. Morris and R. Freeman, J. Magn. Reson. 29 (1978) 433.
14 W.A. Bubb, K. Kirk and P.W. Kuchel, J. Magn. Reson. 74 (1988) 363.
15 G. Robinson, P.W. Kuchel, B.E. Chapman, D.M. Doddrell and M.G. Irving, J. Magn. Reson. 63 (1985) 314.
16 D.J. States, R.A. Haberkorn and D.J. Ruben, J. Magn. Reson. 48 (1982) 286.
17 T.C. Farrar and E.D. Becker, Pulse and Fourier transform NMR (Academic Press, New York, 1971) p. 20.
18 J.J. Led and H. Gesmar, J. Magn. Reson. 49 (1982) 444.
19 G. Robinson, B.E. Chapman and P.W. Kuchel, Eur. J. Biochem. 143 (1984) 643.
20 J. Villadsen and M.L. Michelsen, Solution of differential equation models by polynomial approximation (Prentice-Hall, NJ, 1978).
21 A.J. Miller, Consulting report VT 81/23, CSIRO division of mathematics and statistics, South Melbourne (1981).
22 D.V. Roberts, Enzyme kinetics (Cambridge University Press, Cambridge, 1977).
23 D.L. Rabenstein, J. Biochem. Biophys. Methods 9 (1984) 277.
24 P.W. Kuchel, Biochem. J. 244 (1987) 247.
25 A.P. Dubrunfaut, C.R. Acad. Sci. 23 (1846) 38.
26 F.H. Westheimer, J. Org. Chem. 2 (1937) 431.
27 R. Bentley and D.S. Bhate, J. Biol. Chem. 235 (1960) 1225.
28 P.H. Fishman, J.W. Kusiak and J.M. Bailey, Biochemistry, 12 (1973) 2540.
29 F.F. Fanin, J.O. Evans, E.M. Gibbs and D.F. Diedrich, Biochim. Biophys. Acta 649 (1981) 189.
30 B. Andersen, Acta Chem. Scand. B. 38 (1984) 415.