Intra-erythrocyte microviscosity and diffusion of specifically labelled [glycyl- α - ^{13}C]glutathione by using ^{13}C n.m.r.

Zoltán H. ENDRE, Bogdan E. CHAPMAN and Philip W. KUCHEL

Department of Biochemistry, University of Sydney, Sydney, New South Wales 2006, Australia

(Received 24 April 1983/Accepted 5 August 1983)

[α - ^{13}C]Glycine was incubated with suspensions of human erythrocytes under special buffer conditions to enrich specifically intracellular glutathione with ^{13}C . The metabolically active cells were then subjected to ^{13}C n.m.r. spectroscopy in which the longitudinal relaxation time(s) (T_1) and nuclear Overhauser enhancement(s) of the free glycine and glutathione were measured. With the appropriate analysis, assuming the molecules to be isotropic rotors, intracellular rotational correlation times were calculated. Using these data together with the Stokes–Einstein equation, viscosity and translational diffusion coefficients were calculated. The results were compared with those from cell lysates and extracts. The cytosolic microviscosity probed by glutathione was only 1.9 ± 0.3 times that of saline, suggesting, therefore, that most enzyme reactions involving this solute are not likely to be diffusion-controlled inside the erythrocyte.

A knowledge of the diffusion rate of intracellular metabolites is necessary for a better understanding of the control of metabolic reactions inside cells. The random diffusion of a molecule will be determined by its size and shape and the viscosity of the medium through which it moves. Studies of intracellular viscosity have employed fluorescence polarization (Wojcieszyn *et al.*, 1981), electron spin resonance (Morse *et al.*, 1979) and ^{13}C n.m.r. spectroscopy (London *et al.*, 1975, 1976). ^{13}C n.m.r. is a non-invasive method of measuring viscosity as the introduction of an isotope label does not perturb the cell as do fluorescent or electron spin probes. The relaxation behaviour of the ^{13}C nucleus can be related to the rotational motion of the molecule incorporating the nucleus. The low sensitivity of the technique may be improved by growing cell cultures or animals on ^{13}C enriched media although this can be a long and costly process.

The tripeptide glutathione (GSH) is present in a number of cells (e.g. liver, kidney and erythrocyte) in high concentration. In human erythrocytes its concentration is approximately 2 mmol/l of cells. Glutathione synthetase (EC 6.3.2.3) catalyses an exchange reaction between free glycine and the glycyl residue of GSH in suspensions of erythrocytes in the absence of glucose (York *et al.*, 1982). Addition of glucose inhibits the reaction. In the

present work erythrocyte suspensions in phosphate buffer were incubated with [α - ^{13}C]glycine which was incorporated into glutathione to a concentration of 1 mmol/l of cells after 4 h at 37°C. This process specifically labels a single carbon atom in glutathione and provides a sufficiently large [glycyl- α - ^{13}C]GSH signal to allow rapid measurement of the longitudinal relaxation time(s) (T_1) and nuclear Overhauser enhancement(s) (n.o.e.).

We have measured ^{13}C T_1 and n.o.e. for glycine and the glycyl residue of GSH in cells, lysates and iso-osmotic saline solutions and determined the viscosity of the intracellular space occupied by these molecules. These measurements allow an estimate to be made of the translational diffusion coefficients of free glycine and GSH inside the cells.

Experimental

Preparation of cells and lysates

Human erythrocytes were prepared from freshly drawn venous blood by washing three times in iso-osmotic saline in $^1\text{H}_2\text{O}$ at 4°C and once in iso-osmotic sodium phosphate buffer, pH 7.4, containing antibiotics [sodium penicillin G (75 $\mu\text{mol/l}$), streptomycin sulphate (75 $\mu\text{mol/l}$) and amphotericin B (1.1 mg/l)] in $^1\text{H}_2\text{O}$. They were gassed with O_2/CO_2 (19:1) for 1 min and the haematocrit adjusted to 0.8. They were then incubated with [α - ^{13}C]glycine, (Merck, Sharp and Dohme,

Abbreviations used: GSH, reduced glutathione; n.o.e., nuclear Overhauser enhancement.

Montreal, Canada) (25 mmol/l of cell suspension) at 37°C for 4 h. After incubation the cells were washed twice with Krebs bicarbonate buffer plus antibiotics (as above) in $^2\text{H}_2\text{O}$ containing glucose (10 mmol/l). The cells were regassed and haematocrit adjusted to 0.8 for cell samples (a cell concentration at which minimal sedimentation occurs). To minimize dilution of the intracellular milieu by extracellular fluid, lysates were prepared by sonication of haematocrit 0.95 cell samples (the maximum haematocrit easily attained with little risk of premature cell lysis), at 40 W for 10 s using a Branson Sonifier Model B-15P (Branson Sonic Power Co., Danbury, CT, U.S.A.). Samples were stored at 4°C prior to n.m.r. measurements. Just before n.m.r. measurements 1.8 ml of sample was placed in a 10 mm (outside diameter) n.m.r. tube and thermally equilibrated at 37°C in a heating block.

^{13}C n.m.r. spectroscopy

^{13}C n.m.r. spectra were obtained at 100.62 MHz using a Bruker WM400 spectrometer in the Fourier mode at 37°C.

The free induction decays were collected in 16384 data points using a sweep width of 22250 Hz. Low power continuous wave proton decoupling at either the GSH-glycyl or glycine $\alpha\text{-CH}$ resonance was used during T_1 and n.O.e. measurements of the respective nucleus. While doubling the number of experiments, this enabled the temperature of the samples to be maintained at 37°C. The temperature was measured with a thermocouple.

T_1 values were determined using the $180^\circ\text{-}\tau\text{-}90^\circ$ pulse sequence (Freeman & Hill, 1971) with a delay of $5 T_1$ between each acquisition. The n.O.e. was measured using the gated decoupling method (Freeman *et al.*, 1972) with a delay of $5 T_1$ for spectra with n.O.e. and a decoupler off time of $8 T_1$ (Opella *et al.*, 1976) for spectra without n.O.e. A total of 128 transients for GSH-glycyl and 16 transients for glycine were averaged for both T_1 and n.O.e. experiments. The glycine $\alpha\text{-C}$ peak was used as an internal reference for chemical shifts, which are reported in p.p.m. from external tetramethylsilane (42.8 p.p.m. upfield from glycine). T_1 and n.O.e. measurements were performed on cells, cell lysates, an extract of lysates containing $[\alpha\text{-}^{13}\text{C}]\text{-glycine}$ and $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ and on saline/ $^2\text{H}_2\text{O}$ to which $[\alpha\text{-}^{13}\text{C}]\text{glycine}$ had been added. Difficulty in isolating ^{13}C -enriched GSH from the lysate extract precluded measurement of its T_1 and n.O.e. in saline/ $^2\text{H}_2\text{O}$.

Addition of known amounts of $[\alpha\text{-}^{13}\text{C}]\text{glycine}$ to lysates and comparison of $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}/[\alpha\text{-}^{13}\text{C}]\text{glycine}$ intensity ratios enabled estimation of the $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ concentration, which was about 1 mmol/l of cells ($\approx 50\%$ of total GSH).

Viscosity measurements

Viscosity measurements were obtained using an Ostwald viscometer in a water bath equilibrated at 37°C. Distilled water was used as the reference compound for calibration. The viscosities of saline/ $^2\text{H}_2\text{O}$, lysate (prepared from haematocrit 0.95 cells) and a lysate extract were determined. The lysate extract was prepared by thermally denaturing haemoglobin and other proteins, in a boiling water bath for 10 min, then the proteins were removed by centrifugation.

Results

N.O.e. were measured from the intensity ratio of peaks in spectra obtained under conditions of continuous decoupling and gated decoupling of protons. Fig. 1 shows the GSH-glycyl peak (a) in spectra of cells with (A) and without (B) n.O.e. The peak from free glycine (b) is broad due to the residual coupling of the $\alpha\text{-C}$ protons. The n.O.e. values of GSH-glycyl and glycine $\alpha\text{-C}$ in various solutions are given in Table 1. All n.O.e. values are high, close to the maximum value of 2.988 predicted for carbon atoms relaxed entirely by $^{13}\text{C}\text{-}^1\text{H}$ dipolar interactions in small molecules undergoing rapid rotation (Doddrell *et al.*, 1972). Thus the rotational correlation time τ_r may be determined from the equations relating τ_r to T_1 , T_2 and n.O.e. (Doddrell *et al.*, 1972).

Fig. 1. Measurement of the n.O.e. of intra-erythrocyte $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ (peak a) (A) Spectrum with n.O.e.; (B) spectrum without n.O.e. Experimental conditions are stated in the text.

Table 1. ^{13}C n.m.r. T_1 and n.O.e. values for $[\alpha\text{-}^{13}\text{C}]\text{glycine}$ and $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ in erythrocytes, lysates and extracts. The uncertainty estimates in T_1 measurements represent 1 s.d. obtained from a non-linear least-squares fit on each data set. The coefficient of variation of the n.O.e. measurements is $<10\%$ (cf. Jones *et al.*, 1978) and therefore none of the n.O.e.'s differ significantly from the predicted maximum of 2.988 (see also Bothner-By & Johner, 1978).

	T_1 (s) and n.O.e. (in parentheses) in:			
	Cells	Lysate	Extract	Saline/ $^2\text{H}_2\text{O}$
Glycine	3.29 ± 0.03 (2.92)	2.64 ± 0.04 (2.82)	5.77 ± 0.04 (2.60)	5.80 ± 0.02 (2.97)
GSH-glycyl	0.68 ± 0.01 (2.77)	0.54 ± 0.06 (3.00)	1.31 ± 0.05 (2.92)	—

Fig. 2. Measurement of the T_1 of intra-erythrocyte $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ (peak a) by the inversion-recovery method. Representative spectra at five different τ values are shown.

Fig. 2 shows partially relaxed ^{13}C n.m.r. spectra of the GSH-glycyl $\alpha\text{-C}$ peak (a) at various delay times τ between the 180° and 90° pulses of the inversion recovery pulse sequence. Values of T_1 for the GSH-glycyl and glycine $\alpha\text{-C}$ in various solutions are given in Table 1. The value given for glycine in cells is an approximation as, although the data could be fitted to a single exponential decay, the glycine will equilibrate across the cell membrane in the ratio of extracellular fluid volume:intracellular fluid volume, which is $\sim 1:3$ at haematocrit 0.8.

The relationship between the rotational correlation time τ_r and viscosity η may be derived from the Debye theory of dielectric dispersions in polar liquids (Bloembergen *et al.*, 1948). The correlation time of a sphere of Stokes' radius a in a liquid of viscosity η is

$$\tau_r = \frac{4\pi\eta a^3}{3kT} \quad (1)$$

where T is the absolute temperature and k is the Boltzman constant. The Stokes' radii of glycine and GSH may be determined from the experimentally measured viscosity of saline solution and lysate extract and the values of τ_r obtained from the T_1 measurements on the respective samples. The value of a and the respective values of τ_r may then be used to determine the microviscosity inside the erythrocyte and in lysate. The translational diffusion coefficient D of a molecule of Stokes' radius a is given by the Stokes-Einstein relationship (Stokes, 1856; Einstein, 1906) as

$$D = \frac{kT}{6\pi\eta a} \quad (2)$$

Values of τ_r obtained from the n.m.r. measurements and η and D calculated from eqns. (1) and (2) for glycine and GSH are given in Table 2.

Table 2. Hydrodynamic parameters for $[\alpha\text{-}^{13}\text{C}]\text{glycine}$ and $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ at 37°C

	Cells			Lysate			Saline/ $^2\text{H}_2\text{O}$			Lysate extract		
	$10^{11} \times \tau_r$ (s)	$10^3 \times D$ (cm^2/s)	$10^3 \times \eta$ ($\text{Pa} \cdot \text{s}$)	$10^{11} \times \tau_r$ (s)	$10^3 \times D$ (cm^2/s)	$10^3 \times \eta$ ($\text{Pa} \cdot \text{s}$)	$10^{11} \times \tau_r$ (s)	$10^3 \times D$ (cm^2/s)	$10^3 \times \eta$ ($\text{Pa} \cdot \text{s}$)	$10^{11} \times \tau_r$ (s)	$10^3 \times D$ (cm^2/s)	$10^3 \times \eta$ ($\text{Pa} \cdot \text{s}$)
Glycine	0.71 ± 0.01	0.96 ± 0.03	1.36 ± 0.04	0.89 ± 0.01	0.76 ± 0.03	1.70 ± 0.02	0.40 ± 0.01	1.70 ± 0.02	0.766 ± 0.003	0.403 ± 0.003	1.75 ± 0.02	0.743 ± 0.003
GSH-glycyl	3.45 ± 0.05	0.55 ± 0.12	1.43 ± 0.23	4.40 ± 0.50	0.42 ± 0.13	1.83 ± 0.46	—	—	—	1.78 ± 0.07	1.05 ± 0.06	0.743 ± 0.003

τ_r , rotational correlation time; D , translational diffusion coefficient; η , medium viscosity. The viscosities of saline/ $^2\text{H}_2\text{O}$ and lysate extract were measured using an Ostwald viscometer. All uncertainty estimates represent 1 s.d.; for saline/ $^2\text{H}_2\text{O}$ and lysate extract viscosities these have been calculated directly. For the other measurements these represent ^{13}C T_1 errors propagated through all calculations.

Discussion

The high n.O.e. values obtained for $[\alpha\text{-}^{13}\text{C}]\text{-glycine}$ and $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ are indicative of ^{13}C – ^1H dipole–dipole interactions being the dominant mechanism of longitudinal relaxation of the carbon nuclei. Any other relaxation mechanism would reduce the value of the n.O.e. below the theoretical maximum of 2.988 expected for small molecules undergoing rapid rotation. The relationship between the ^{13}C T_1 and the rotational correlation time τ_r for a molecule undergoing rapid isotropic reorientation is (Kuhlmann *et al.*, 1970)

$$1/T_1 = N\hbar^2\gamma_H^2\gamma_C^2r^{-6}\tau_r \quad (3)$$

where N is the number of directly bonded protons on the ^{13}C nucleus, $\hbar$ is Planck's constant/ 2π , γ_H , γ_C are the respective magnetogyric ratios of protons and ^{13}C nuclei and r is the C–H distance; a value of 0.109 nm was used in this paper (Doddrell *et al.*, 1972).

From the values of τ_r (Table 2) and the measured viscosity of iso-osmotic saline/ $^2\text{H}_2\text{O}$ and lysate extract, the Stokes' radii a for glycine (0.18 nm) and GSH (0.29 nm) were obtained by using eqn. (1). Using these values and the τ_r calculated for glycine and GSH in cells and lysates the internal viscosity of cells and lysates was then determined. The volume ratio GSH/glycine (4.18) obtained from the Stokes' radii is nearly the same as the ratio of specific volumes of GSH and glycine (4.05). This could imply that GSH is tightly folded, such as the figure-of-eight structure reported by Zenin *et al.* (1975). An alternative explanation is that the $[\alpha\text{-}^{13}\text{C}]\text{glycyl}$ of GSH is undergoing internal rotation in addition to the overall rotation of the GSH molecule. This could give rise to a longer T_1 and smaller τ_r than predicted for an isotropic rotor and an underestimation of the Stokes' radius of GSH. For comparison, the Stokes' radius for leucylglycylleucine, calculated from translational diffusion measurements (Cohn & Edsall, 1943), is 0.46 nm. This gives a volume ratio to glycine of 8.28 while the ratio of specific volumes is 4.01, implying that leucylglycylleucine has a partially extended structure in solution.

The ratio of viscosity in cells to that in iso-osmotic saline is 1.78 ± 0.06 with $[\alpha\text{-}^{13}\text{C}]\text{glycine}$ as the viscosity probe, whereas cells:lysate extract is 1.9 ± 0.3 with $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{GSH}$ as the viscosity probe and 1.83 ± 0.06 with $[\alpha\text{-}^{13}\text{C}]\text{glycine}$. This compares with a viscosity ratio for cells:saline of 2.14 using $[\text{histidyl-2-}^{13}\text{C}]\text{haemoglobin}$ as the viscosity probe (London *et al.*, 1975). The effective viscosity as determined by $[\alpha\text{-}^{13}\text{C}]\text{glycine}$ and $[\text{glycyl-}\alpha\text{-}^{13}\text{C}]\text{-GSH}$ is the microviscosity of the space occupied by these molecules and is not necessarily the viscosity 'seen' by a macromolecule such as haemoglobin, which would be subject to more motional con-

straints than a small molecule. While it is necessary to assume that GSH is a rigid isotropic rotor for the calculation of a true τ_r from T_1 , the determination of the ratio of viscosities in cells to that in the lysate-extract or saline requires only that the conformation does not change and may be obtained directly from the ratio of T_1 measurements (eqns. 1 and 3). Knowing these ratios and the absolute viscosity of the lysate extract (measured in the viscometer) the cell viscosity may then be calculated. The direction and approximate magnitude of the viscosity ratios is supported by the cell: saline ratios for [α - ^{13}C]glycine, a molecule which can be reasonably assumed to rotate isotropically. The effective intracellular microviscosity calculated here for cells and lysates is that seen by the free glycine and GSH glycine residues in the concentrated macromolecular solution which forms the cytosol. The viscosity of the aqueous phase of the cytosol is, of course, that measured directly in the lysate extract and is similar to that of iso-osmotic saline/ H_2O . The observation of a higher effective viscosity in lysates than cells is not an artefact due to differences in haematocrit; GSH is purely intracellular and hence the cell T_1 result for GSH is independent of haematocrit, while the small (5%) dilution of the cytosol with extracellular fluid in the lysate should decrease viscosity and hence decrease, not increase, τ_r . The higher effective viscosity in lysates could be due to a disordering of the cytosol and a release of previously membrane-bound molecules into the aqueous phase of the lysate.

The translational diffusion coefficients of glycine and GSH may be determined from eqn. (2). The value is that expected for a molecule undergoing a random three-dimensional walk in an unbounded system. For intracellular glycine $10^5 D$ (cm^2/s) was calculated to be 0.96 at 37°C . This may be compared with values expected for other small particles such as the hydrated lithium ion (0.91; Andrasko, 1976) and water (2.0; Mills, 1973) in a medium of viscosity $1.36 \text{ mPa} \cdot \text{s}$ at 37°C . The value obtained for GSH is an upper limit as, although the relative viscosity may be determined by using an 'experimental' Stokes' radius and isotropic rotational correlation time, the translational diffusion coefficient depends on the absolute value of the Stokes' radius. This has probably been underestimated due to internal rotation of the GSH [α - ^{13}C]glycyl residue, giving a smaller τ_r than expected for an isotropic rotor. The value of $10^5 D$ for GSH, 0.55, may be compared with the expected value of 0.36 for leucylglycylleucine using a Stokes' radius of 0.46 nm.

These diffusion coefficients are, as mentioned above, for isotropic unbounded diffusion. A molecule undergoing diffusion inside a cell moves freely until it reaches the membrane, where it is

deflected back into the cytosol. The apparent diffusion coefficient measured from the observation of 'tagged' molecules in closed compartments will vary with the time interval between measurements of their position. At short time intervals the molecules will appear to have the same diffusion coefficient as in the unrestricted case, governed solely by their size, shape and the viscosity of the medium. At longer observation intervals the molecules will appear to have a smaller diffusion coefficient because they are deflected back towards their starting position after rebounding from the membrane. However, for a metabolite moving towards an enzyme the 'unrestricted' diffusion coefficient will determine the time taken for the metabolite to reach enzyme, not the apparent diffusion coefficient which is measured experimentally and which is time-dependent.

In conclusion, measured rotational diffusion coefficients and τ_r of cytosolic solutes will be influenced little by the cell membrane in contrast to translational diffusion coefficients. Therefore, translational diffusion coefficients calculated from the rotational correlation time using eqns. (1) and (2), which apply to the isotropic unbounded case, yield the true translational diffusion coefficient; given, of course, the assumptions of spherical molecules. Direct measurement of the translational diffusion coefficient by, say, pulsed field gradient n.m.r., yields an apparent value which may not necessarily be relevant to the question of whether diffusion limits the rate of cytosolic enzyme-catalysed reactions in whole cells. The value of D for glutathione in the present work suggests that the motion of the molecule is not severely restricted, and given the magnitude of the 'on' rate constants estimated for glutathione reductase (Staal & Veeger, 1969) it is unlikely that diffusion control of this reaction will arise in the human erythrocyte. However, Brouwer & Kirsch (1982) studied chymotrypsin-catalysed reactions in sucrose and Ficoll solutions with viscosities up to 3.2 times that of the buffer and noted a significant effect on the rate of some of the esterase reactions. Therefore, it is still conceivable that intraerythrocyte viscosity may retard a very fast reaction involving glutathione, namely that catalysed by glutathione peroxidase.

Project grant support to P. W. K. and B. E. C. and a post-graduate scholarship to Z. H. E. were given by the Australian National Health and Medical Research Council. Mr. B. T. Bulliman and Mr. W. G. Lowe are thanked for computing and technical assistance, respectively.

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