THE INCORPORATION OF ^{2}H-LABELLED GLYCINE INTO THE GLUTATHIONE OF INTACT HUMAN ERYTHROCYTES STUDIED BY ^{1}H SPIN-ECHO FOURIER TRANSFORM NMR

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1. Introduction

Although the concentration of glutathione (γ-L-glutamyl–L-cysteinyl–glycine, GSH) in human erythrocytes is generally found to be ~2 mM [1,2], it is in a dynamic state, continually being degraded to and resynthesized from its constituent amino acids. The details of the degradation and synthesis reactions are the subject of considerable research [3,4], as is the mechanism by which the GSH level is regulated [1]. Much of this research has focussed on the individual steps in the overall process using enzyme preparations, although GSH dynamics in whole cells have also been studied by following the incorporation of radioactively-labelled amino acids [5,6].

GSH is considered to be essential for maintenance of the structural integrity of the erythrocyte [7], being involved in the protection of protein thiol groups against oxidation, the catalysis of sulfhydryl–disulfide exchange reactions, and the detoxification of foreign compounds [4]. In assessing the GSH status of the cell, not only is the GSH level of interest but also the capacity of the cell for GSH synthesis. Although this information can be obtained from whole cell studies using radioactively labelled amino acids, rather elaborate separations are necessary prior to the measurement step. Here we show that intracellular GSH dynamics can be monitored directly by ^{1}H NMR spectroscopy by following the incorporation of ^{2}H-labelled glycine. The results also show that information about other cellular reactions can be obtained simultaneously from the same set of measurements.

Abbreviations: NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulphonic acid; GSH, reduced glutathione; FT, Fourier transform

2. Experimental

The erythrocytes were prepared from freshly drawn venous blood which was collected in vacutainers (Becton, Dickson and Co.) containing EDTA solution. The whole blood was centrifuged at 5000 rev./min at 4°C for 15 min, and the packed cells were washed 4 times with Krebs-Ringer [8] in D_2O. To identify the glycine resonance, 1 part packed cells were suspended in 10 parts of a D_2O solution containing 0.154 M NaCl, 0.005 M glucose and 0.005 M glycine. After 15 h incubation, the cells were isolated by centrifugation. To study the incorporation of ^{2}H-labelled glycine (D_5-glycine, 98% deuterated from Merk, Sharp and Dohme) into the intracellular GSH, packed cells were suspended in an equal volume of D_2O solution containing 0.154 M NaCl, 0.005 M glucose and 0.050 M D_5-glycine. After incubation for 1.25 h, the cells were isolated by centrifugation. Packed cells (~0.5 ml) were transferred to an NMR tube, which was then left in the NMR spectrometer continuously for a 24 h period. An alternative procedure which we also have used involves incubation of the 1:1 suspension of erythrocytes in D_5-glycine solution at 37°C in a constant temperature water bath. Cells are then isolated from portions of this suspension as a function of time, and NMR spectra are obtained for the packed cells from each portion.

^{1}H NMR spectra were measured at 400 MHz on a Bruker WH-400/DS spectrometer by the spin-echo Fourier transform technique ($90°-\tau_2-180°-\tau_2-$ acquisition, with τ_2 generally = 0.060 s) [9,10]. Spectra were measured at 25°C on ~0.5 ml of packed cells contained in 5 mm o.d. NMR tubes. The free induction decay was collected in 8 K of data points with an acquisition time of 0.819 s. Quadrature detection was used with spectral widths of 5000 Hz, and 300 transients were collected for all the spectra. Chemical shifts are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulphonic acid (DSS), based on the resonance for the α-CH_2 protons of the Gly residue of GSH having a chemical shift of 3.76 ppm.

^{2}H NMR spectra were measured at 61.4 MHz on a Bruker WH-400/DS spectrometer. Spectra were obtained by the single pulse sequence on ~5 ml packed erythrocytes in a 15 mm o.d. NMR tube. The erythrocytes were prepared as above with the exception that H_2O was used as solvent. Chemical shifts were measured relative to the HDO signal (4.80 ppm).

3. Results and discussion

The standard ^{1}H NMR spectrum for intact erythrocytes consists of broad, rather featureless envelopes due to overlap of the multitude of resonances, the majority of which are from hemoglobin. Buried within the envelope are the resonances for GSH. Figure 1A shows that, with the spin-echo technique,

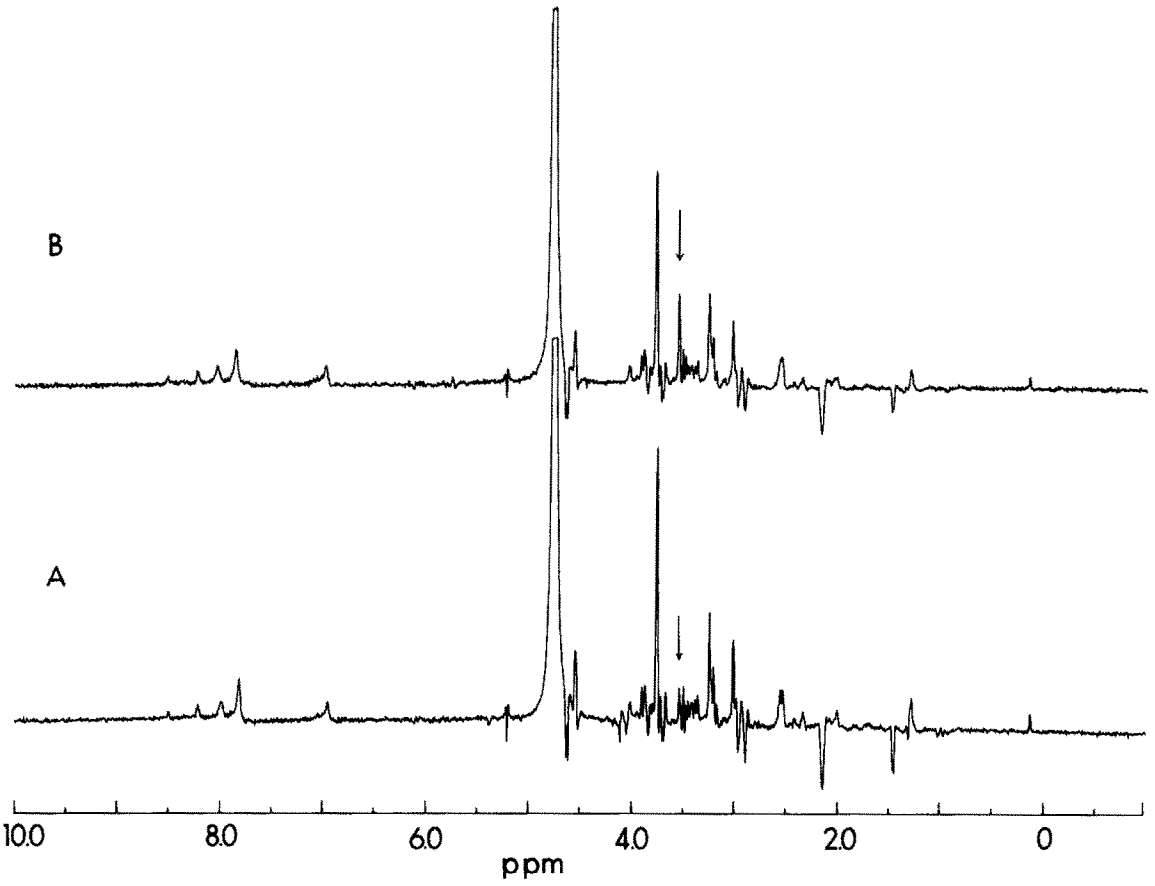

Fig.1. 400 MH_Z ^{1}H spin-echo FT NMR spectra (τ_2 = 0.060 s) of intact human erythrocytes at 25°C. (A) Packed cells washed 4 times with D_2O solution containing 0.154 M NaCl and 0.005 M glucose. (B) Packed cells washed as in (A) and then incubated in D_2O solution containing 0.154 M NaCl, 0.005 M glucose and 0.005 M glycine.

most of the hemoglobin resonances can be selectively eliminated, the hemoglobin resonances are eliminated because of their short spin–spin relaxation times relative to τ_2 in the spin-echo sequence [10,11].

The resonances that remain in the 0–6 ppm region are due to the carbon-bonded protons of some of the small molecules in the intracellular region [11]. Those at 4.55 and 2.93 ppm are due to the α-CH and β-CH_2 protons of the Cys residue of GSH, that at 3.76 ppm is due to the α-CH_2 protons of the Gly residue, and those at 2.55 and 2.15 ppm are due to the γ-CH_2 and β-CH_2 protons of the Glu residue. In the spin-echo experiment, those protons which are split by proton–proton spin–spin coupling can give rise to negative resonances and to resonances which are out-of-phase, e.g., the resonances for the β-CH_2 protons of the Cys residue and the γ-CH_2 protons of the Glu residue [10]. The resonance at 3.54 ppm is assigned to the α-CH_2 protons of free glycine on the basis of the increase observed for this signal for packed cells which have been suspended in a glycine-containing saline solution (fig.1B). The signal at 3.25 ppm is from the 9 equivalent methyl protons of ergothioneine, those at 5.22 and 4.63 ppm are from the anomeric protons of α-D-glucose and β-D-glucose, respectively, and that at 1.28 ppm is from the methyl protons of lactic acid. The methyl resonance of lactic acid is a positive singlet, rather than an inverted doublet as would be expected for the conditions used in these spin-echo experiments, because C(2) of the lactate produced by glucose metabolism in D_2O is deuterated [11].

The results in fig.1 show that the free glycine in intact erythrocytes can be observed by ^1H NMR, and that glycine can be introduced into the cell by incubation in a glycine solution. Figure 2 shows, as a function of time, a portion of the ^1H spin-echo spectrum for packed erythrocytes which had been incubated in a solution containing D_5-glycine. Initially, there is no change in the spectrum. As time passes, however, the resonance at 3.54 ppm increases while that at 3.76 ppm decreases, indicating that the added D_5-glycine has become part of the free glycine pool and is being used in the synthesis of GSH. In fig.3, time courses are plotted for these two signals, using the methyl resonance of ergothioneine as an internal standard.

The results in fig.2 and 3 indicate that the resonance at 3.54 ppm increases in intensity more than the resonance at 3.76 ppm decreases in intensity. This may be due to the substitution of D_5-glycine for other forms of glycine in the erythrocyte or to different amounts of attenuation of the resonances for the α-CH_2 protons of free glycine and the Gly of GSH due to different spin–spin relaxation times [10]. The ratios of the intensity of the GSH resonances at 4.55, 2.55 and 2.15 ppm to the intensity of the ergothioneine resonance remain constant, indicating

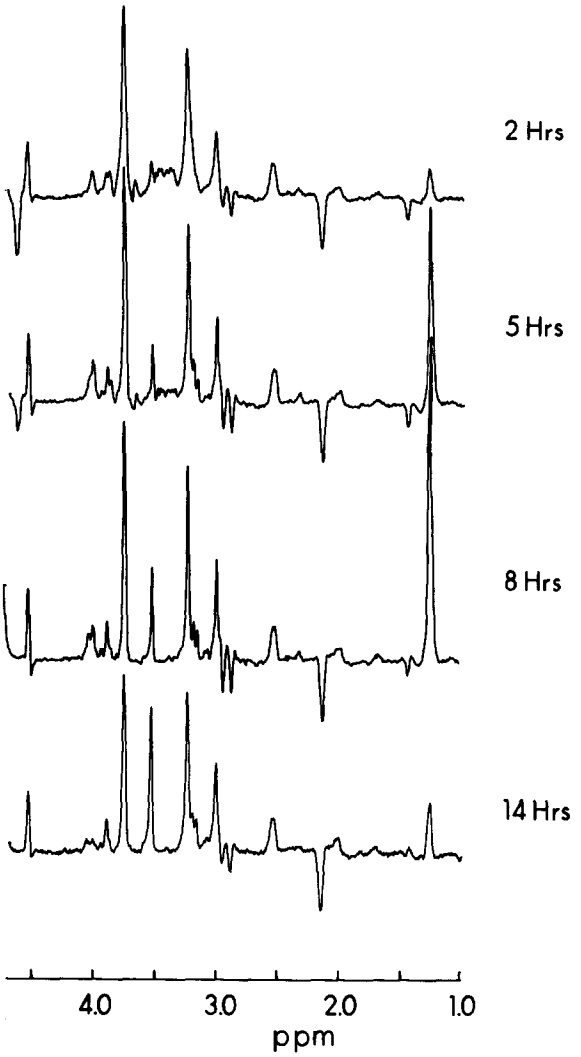

Fig.2. Portions of the 400 MH_Z ^1H spin-echo FT NMR spectra (τ_2 = 0.060 s) of intact human erythrocytes which have incubated in a saline–glucose solution containing 0.05 M D_5-glycine. The times are from the time of separation of the cells from the incubation mixture.

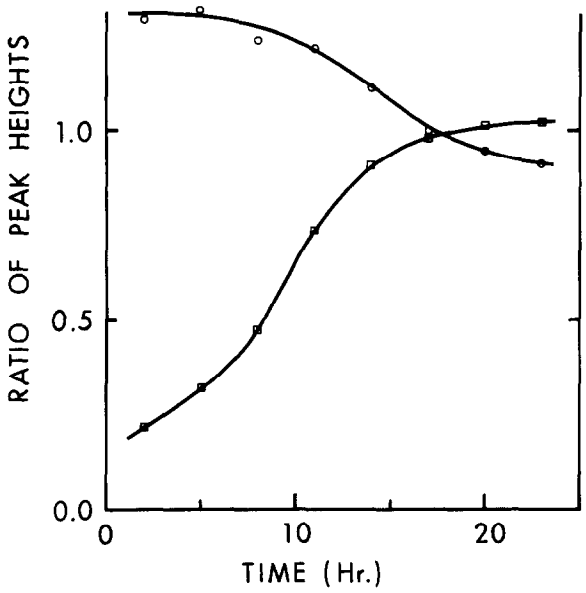

Fig.3. Time courses for the decrease in the intensity of the α-CH_2 resonance of the Gly residue of GSH (○) and the increase in the intensity of the free Gly resonance ([]). The results are presented as the ratios of the heights of these resonances to the resonance at 3.25 ppm for the methyl protons of ergothioneine, which is assumed to remain constant throughout the experiment.

little if any change in the total GSH concentration during the time course experiment.

We have also done similar experiments to study the incorporation of ^{2}H-labelled glutamic acid. Incorporation is observed, as indicated by decreases in the intensity of the resonances for the β-CH_2 and γ-CH_2 protons of the Glu residue and increases in the intensity of resonances for the corresponding protons of free glutamic acid. As reported [6] using radioactively-labelled glutamic acid, we find the rate of incorporation of glutamic acid to be considerably slower than that of glycine.

We have also attempted to follow the incorporation of D_5-glycine into the intracellular GSH by deuterium NMR. After incubation of packed erythrocytes in a solution containing D_5-glycine, a signal is observed at 3.54 ppm in the ^{2}H NMR spectrum, indicating that D_5-glycine has entered the cell. A signal appears with time in the region of 3.76 ppm, indicating incorporation into GSH, however both it and the signal at 3.54 ppm are so broad and their separation so small (13.5 Hz) that it was not possible to obtain quantitative information about the time course by deuterium NMR.

The results in fig.2 and 3 demonstrate that the capacity of erythrocytes for GSH synthesis can be followed by ^{1}H NMR. The spectra also indicate that information about other cellular processes can be obtained simultaneously. For example, the resonances for the anomeric protons of α-D-glucose and β-D-glucose are clearly resolved in fig.1. During the time course experiment, these decrease in intensity as the glucose is metabolized, making it possible to measure the rate of glucose metabolism. At the same time, the rate of lactic acid production can be followed by the intensity of its signal at 1.28 ppm [11]. The results in fig.2 also show that, after the glucose supply has been exhausted as indicated by the disappearance of the signal from the anomeric proton of β-D-glucose, the methyl resonance of lactic acid decreases in intensity. This is presumably due to ^{2}H replacement of methyl hydrogens as a result of the pyruvate–lactate equilibration. Also, the signal at 2.93 ppm indicates that the glutathione is in the reduced form throughout the experiment, even after the glucose has all been metabolized.

The measurements presented here have been made with cells in which most of the intracellular water has been replaced by D_2O. We have shown, however, that D_2O substitution is not necessary when high frequency spectrometers (e.g., 400 MHz) are used [12]. At the high frequency, the spin–spin relaxation time of the resonance for the intracellular H_2O is short relative to those of GSH and other small molecules in the erythrocyte so that its signal can be completely eliminated by using a sufficiently long τ_2.

It is important to note that the entire spectrum is obtained in the pulsed NMR experiment, giving simultaneously all the information discussed above. Thus, ^{1}H spin-echo NMR appears to be a powerful method for simultaneously studying GSH synthesis and other metabolic reactions in erythrocytes.

Acknowledgements

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