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PROTON NMR SPECTROSCOPIC STUDIES OF DIPEPTIDASE IN HUMAN ERYTHROCYTES

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In studies on human erythrocyte metabolism in situ, high resolution (400 MHz) ^{1}H spin-echo NMR spectroscopy was used to follow the time dependence of hydrolysis of glycylglycine and L-cysteinylglycine in intact cells and their lysates. The concentration dependence of the hydrolysis of L-cysteinylglycine was described by a rectangular hyperbola with K_m, 3.5 ± 0.6 mmol/ℓ lysate and V_{max}, 64.2 ± 3.2 mmol/ℓ lysate/h. We demonstrated that glycylglycine readily enters the erythrocyte and we introduce a means of analysing the data from the coupled reaction sequence; the sequence consists of transport followed by enzyme catalysed hydrolysis.

Human erythrocytes contain proteolytic enzymes in both the membrane and the cytosol; most reports on the subject have dealt with activities associated with the membrane (1,2). The few reports on cytosolic proteinases and peptidases in the mature erythrocyte suggest that there is a range of endopeptidase, dipeptidylaminopeptidase and aminopeptidase activities (3). The function of proteolytic enzymes in the erythrocyte has not been fully elucidated.

It has been reported (4) that rabbit erythrocytes contain a functional γ-glutamyltranspeptidase-cyclotransferase pathway accounting for the turnover of erythrocyte glutathione. This cycle is dependent on the presence of a dipeptidase to hydrolyse the L-cysteinylglycine formed. More recent work (5), however, has shown that neither rabbit nor human mature erythrocytes contain γ-glutamyltranspeptidase in preparations that are free of white cells; thus the presence of dipeptidase activity appears to be unrelated to glutathione turnover in these cells. It is possible that the dipeptidase activity is the residuum of activity present in the differentiating cell. As dipeptides are not, in general, substrates for

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metabolic reactions the dipeptidase activity in the erythrocyte could serve the role of assisting in the protection of the organism against the loss of amino acids from the metabolic pool (6).

Dipeptides are hydrolysed at a much lower rate by intact erythrocytes than by erythrocyte lysates (6). The hydrolysis of dipeptides by intact cells appears to be rate limited by the transport of the dipeptide into the erythrocytes. In this communication we show that Nuclear Magnetic Resonance spectroscopy (NMR) can be easily adapted to studying dipeptidase activity in intact erythrocytes and lysates. We also show how NMR has been used to monitor the transport (and hydrolysis) of glycylglycine in intact erythrocytes, using an extension of an earlier procedure (7).

MATERIALS AND METHODS

L-Cysteinylglycine was obtained from Vega Biochemicals, Tucson, Arizona, USA, glycylglycine from Sigma, Saint Louis, Missouri, USA, desferrioxamine mesylate from Ciba Laboratories, Horsham, West Sussex, UK and [2,3,4,6,6-$^{2}H_5$]D-glucose from Merck, Sharp and Dohme, Point Claire-Dorval, Quebec, Canada. All other reagents were of AR grade.

Human erythrocytes were prepared from freshly drawn venous blood by washing twice in isotonic saline in $^{1}H_2O$ and once in Krebs bicarbonate (8) in $^{2}H_2O$. The Krebs solution contained antibiotics (sodium penicillin G 27 mg/ℓ, streptomycin sulfate 50 mg/ℓ and amphotericin B 2.5 mg/ℓ) and 10 mM nicotinamide (9). The hematocrit (Hc) of the cell suspensions was measured and they were then lysed by freezing and thawing.

L-Cysteinylglycine was reduced with mercaptoethane sulfonic acid and purified on a Dowex 2-X8 anion exchange resin before use. The paramagnetic ion Fe^{3+} was liganded by addition of $FeCl_3$ to desferrioxamine (1:1 mole ratio). In some experiments the rate of hydrolysis of dipeptide by lysate prepared from Hc = 0.8 cells was too rapid to follow by NMR. In these cases the lysate was diluted with Krebs bicarbonate solution to reduce the activity.

Just prior to NMR measurements, 0.5 ml of lysate or cells was placed in a 5 mm o.d. NMR tube and heated to 37°C. Various reagents were then added (see text). On addition of reagents the samples were placed in a heating block at 37°C for 1 min followed by NMR measurements. The zero time of the reaction was taken as the time reagents were added. Measurements were started within 3 h of drawing blood.

^{1}H NMR spectra were measured at 400 MHz in the Fourier mode using a Bruker WM400 spectrometer at 37°C. The inversion recovery spin-echo sequence (10) $180°$-τ-$90°$-τ-$180°$-τ with τ = 0.06 s, and a repetition time of 1.0 s was used. A total of 64-256 transients were averaged using a sweep width of 5000 Hz and 8192 data points. Chemical shifts are quoted relative to tetramethylsilane (TMS)/CCl_4 present in a small capillary inside the NMR tube. Glycine concentrations were determined from the intensity ratio of the glycine αCH_2 resonance to the TMS signal or the ergothioneine quaternary amine methyl signal. Calibration curves of glycine concentration vs intensity ratios were prepared by addition of known amounts of glycine to lysate samples.

RESULTS AND DISCUSSION

Expanded spectra of erythrocyte lysate with 10 mM L-cysteinylglycine, at various incubation times are shown in Fig. 1. The group of resonances marked a arise from the glycyl of L-cysteinylglycine, showing an AB pattern, and the glycyl of glutathione. Peak b is free glycine, peak c the C-5 proton of $^{2}H_{5}$-glucose and peak d the quaternary amine methyl signal of ergothioneine. Signals from the β-CH_2 of cysteinyl and free cysteine are upfield of the ergothioneine peak; assignment in this region of the spectrum is difficult as there is overlap of peaks with lysate resonances. Peak e is the β-CH_2-glutamyl signals of glutathione.

It is evident from the spectra that the glycyl peak of L-cysteinyl-glycine fell and the free glycine peak increased with time. From these spectra, plots of free glycine concentration vs time gave initial velocities for the hydrolysis of L-cysteinylglycine by the lysate. The dependence of

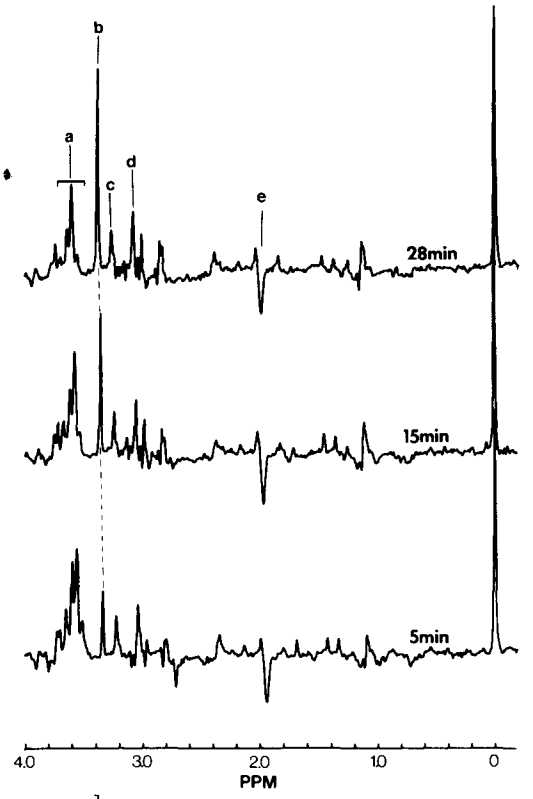

Fig. 1. 400 MHz ^{1}H NMR spectra of L-cysteinylglycine (10 mM) in erythrocyte lysate.

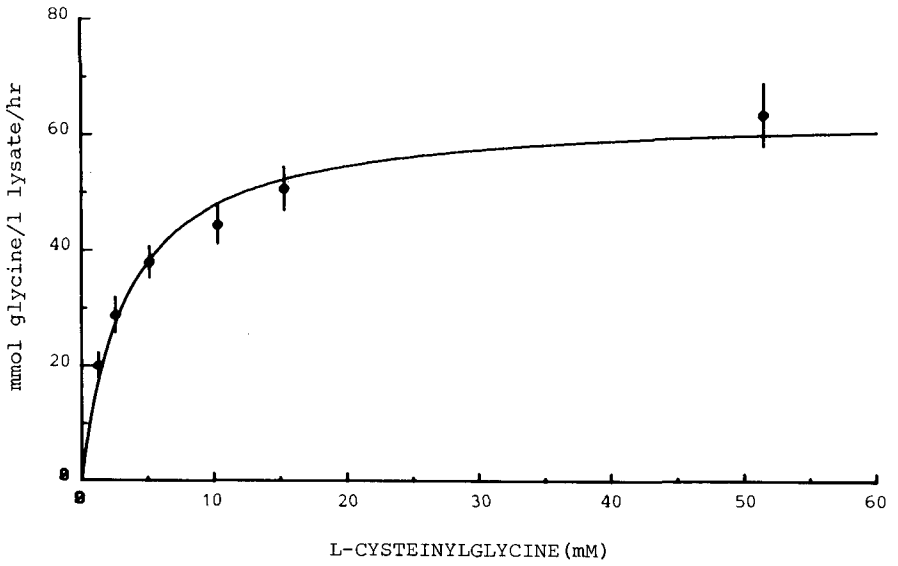

Fig. 2. The rate of release of glycine versus L-cysteinylglycine concentration in erythrocyte lysate at 37°C.

the rate of release of free glycine on the concentration of L-cysteinylglycine is shown in Fig. 2. The data set was analysed by non-linear regression (11) on the Michaelis-Menten equation and yielded an apparent K_m of 3.5 ± 0.6 mM and a V_{max} of 64.2 ± 3.2 mmol/ℓ lysate/h. The peak intensities of glycine relative to a TMS reference were converted to concentrations by reference to a glycine calibration curve and corrected for hematocrit and dilution.

The turnover time of glutathione in human erythrocytes has been estimated to be 4 to 5 days (12). Synthesis of the 2 mmol/ℓ cells of glutathione therefore has a net rate of $\sim 2 \times 10^{-5}$ mol/ℓ/h, which is 3000 times less than the maximal velocity of the L-cysteinylglycine (dipeptidase) reaction (Fig. 2). Since in vivo L-cysteinylglycine is below the detection limit of our NMR experiments the concentrations of the free species must be less than ∿ 0.1 mM; at this substrate concentration the rate of the dipeptidase catalysed reaction would be 1.8 mmol/ℓ cells/h which is still greatly in excess of the rate of net GSH turnover in erythrocytes, but not of that in rat kidney (13). Only at an L-cysteinylglycine concentration of 1.1×10^{-6} M (which may be less than that of the enzyme concen

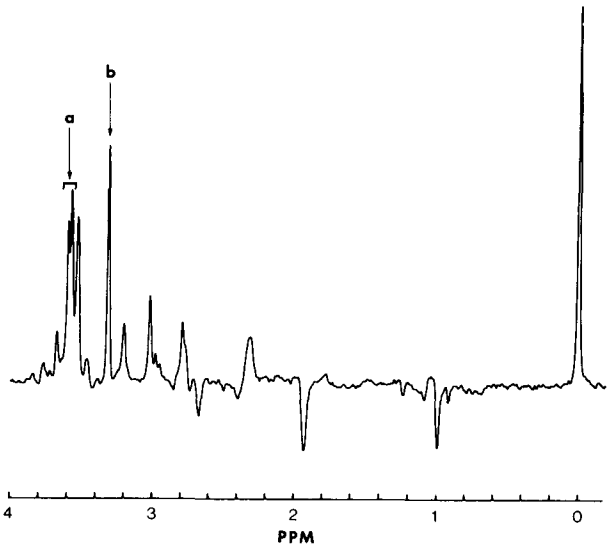

Fig. 3. 400 MHz ^{1}H NMR spectrum of glycylglycine (8 mM) in a suspension of human erythrocytes (Hc = 0.8) after incubation at 37°C for 4 h.

tration) would the enzyme, assuming no interference by effectors, become rate limiting. Therefore the high activity of the enzyme in erythrocytes raises the possibility that it has a role in addition to L-cysteinylglycine turnover for glutathione synthesis.

Fig. 3 shows an expanded ^{1}H NMR spectrum of glycylglycine in a suspension of intact erythrocytes (Hc = 0.8) washed in Krebs bicarbonate buffer in $^{2}H_2O$. The peak marked a is from the glycine residues of glycylglycine and that marked b from free glycine. The transport of glycylglycine can be followed relatively easily using ^{1}H NMR. The method depends on molecules inside and outside the cell having different specific intensities (7). In the present system this was arranged by changing the bulk magnetic susceptibility of the outside medium, which had the effect of enhancing magnetic field gradients outside the cell and broadening and suppressing signals from molecules outside the cell. This was achieved by the addition of the paramagnetic complex Fe^{3+}-desferrioxamine which is known to not cross the cell membrane (14). The complex is effective at low concentration ($\sim$ 1 mM) and does not alter the metabolic properties of the cell.

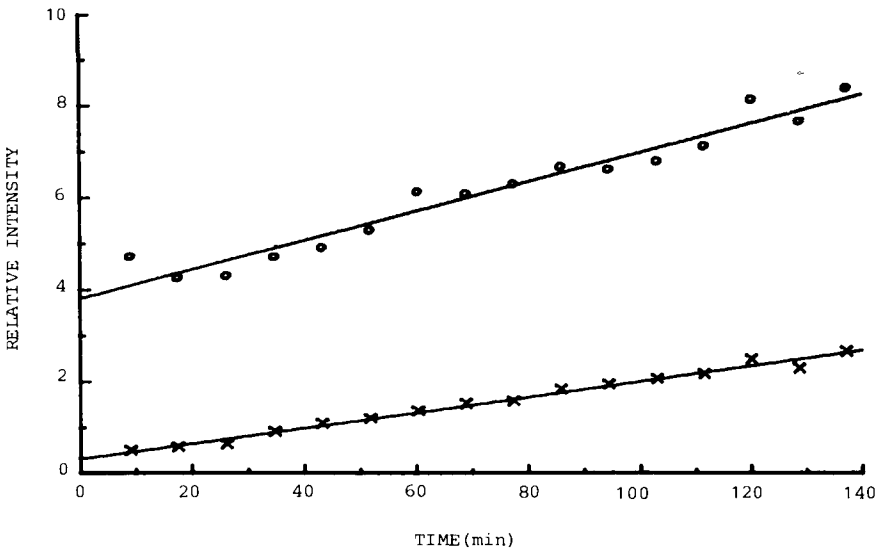

Fig. 4. Plots of intensity relative to ergothioneine of the glycylglycine (O) and free glycine (X) signals with time. Sample conditions; Hc = 0.8, 10 mM glycylglycine and 1 mM Fe^{3+}-desferrioxamine.

Fig. 4 shows the signal intensity of glycylglycine and free glycine as a function of time. The glycylglycine peak increased as the peptide was transported into the more magnetically homogeneous intracellular environment. The free glycine peak also increased as the intracellular glycylglycine was hydrolysed by the dipeptidase. By reference to a calibration curve the rate of glycine release was calculated to be 1.7 ± 0.1 mmol/ℓ cells/h. Under identical conditions of hematocrit and substrate concentration the rate of glycine release by lysed cells was 18.9 ± 0.8 mmol/ℓ lysate/h. Hydrolysis of glycylglycine by intact cells was therefore approximately 11 times slower than by lysates. The rate of transport of glycylglycine into the cells was faster than its hydrolysis, and was quantified by using the following expression for extracellular glycylglycine concentration:

$$[\text{glycylglycine}]_{\text{outside}} = (4g_1 + g_2 - 2g_1\xi_{1,i})/(\xi_{2,o} - 2\xi_{1,i}) \quad ,$$

where g_1 and g_2 are the glycine and glycylglycine intensities in the spectrum and $\xi_{1,i}$ and $\xi_{2,o}$ are the coefficients which relate intracellular glycine and extracellular glycylglycine to peak intensity; these values were obtained from a standard curve for glycine in a lysate, and from the initial combined glycylglycine peaks at the commencement of the reaction. The formula was

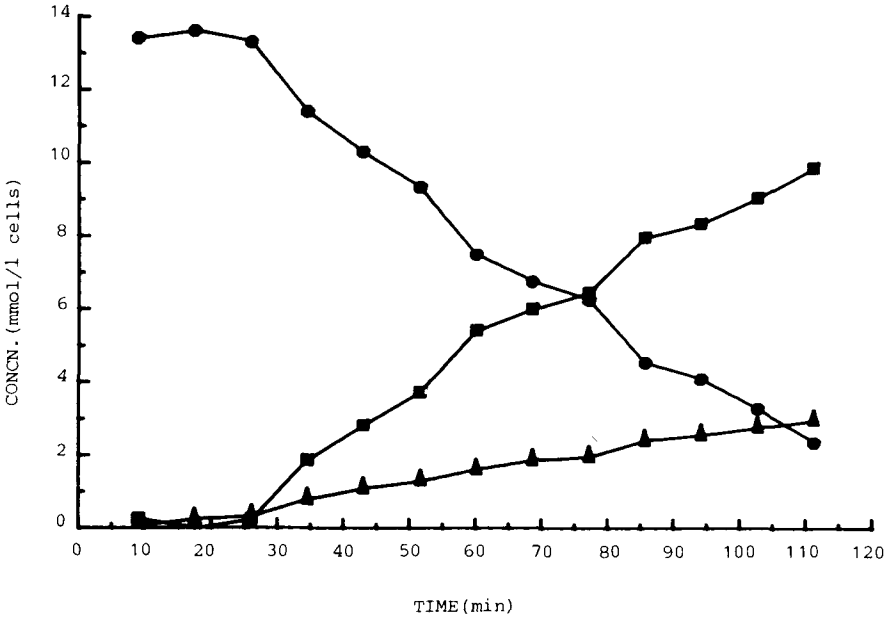

Fig. 5. Extracellular (●), intracellular glycylglycine (■) and intracellular glycine (▲) in a suspension of erythrocytes (Hc = 0.8) with 1 mM Fe^{3+}-desferrioxamine. Data from Fig. 4 analysed according to the formula in the text.

developed from the expression for the conservation of glycylglycine mass and assumed that $\xi_{2,i} = 2\xi_{1,i}$ (which was experimentally verified) and efflux from the cell is negligible during the time of the experiment.

Fig. 5 shows the time course of the overall coupled reaction analysed from the data of Fig. 4 using the previous expression. From linear regression analysis the rate of glycylglycine influx after 30 min was 7.2 ± 0.3 mmol/ℓ cells/h; by taking the difference between the concentration of extracellular glycylglycine and intracellular free glycine the concentration of intracellular glycylglycine was inferred. Interestingly, the transport rate of the dipeptide is many times that of the corresponding amino acid, glycine (15), a feature that has been demonstrated for amino acid and peptide absorption in the gut (16). The total physiological role of erythrocyte peptidases is unknown, but in view of the rapid entry of glycylglycine it, and other peptides released in plasma during hormone activation (e.g. angiotensin I → angiotensin II) or during inflammation and clot dissolution, may possibly enter erythrocytes and be broken down to the constituent amino acids.

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REFERENCES

1. Moore, G.L., Kocholsty, W.F., Cooper, D.A., Gray, J.L. and Robinson, S.L. (1970) Biochem. Biophys. Acta. 212, 126.
2. Tokes, Z.A. and Chambers, S.M. (1975). Biochem. Biophys. Acta. 389, 325.
3. Pontremoli, S., Milloni, E., Salamino, F., Speratore, B., Michetti, M., Benatti, A., Morelli, A. and de Flora, A. (1980). Eur. J. Biochem. 110, 421.
4. Palekar, A.G., Tate, S.S. and Meister, A. (1974). Proc. Nat. Acad. Sci. USA. 71, 293.
5. Srivastava, S.K., Awasthi, Y.C., Miller, S.P., Yoshida, A. and Beutler, E. (1976). Blood 47, 645.
6. Krzysik, B.A. and Adibi, S.A. (1977). Amer. J. Physiol. 233, E450.
7. Brindle, K.M., Brown, F.F., Campbell, I.D., Grathwohl, C. and Kuchel, P.W. (1979). Biochem. J. 180, 37.
8. Krebs, H.A. and Henseleit, K. (1932). Z. Physiol. Chem. 210, 33.
9. Kuchel, P.W., Jarvie, P.E. and Conyers, R.A.J. (1982). Biochem. Med. 27, 95.
10. Rabenstein, D.L. (1978). Analytical Chem. 50, 1265.
11. Osborne, M.R. (1976). Aust. Math. Soc. B. 19, 343.
12. Dimant, E., Landsberg, E. and London, I.M. (1955). J. Biol. Chem. 213, 76.
13. Orlowski, M. and Meister, A. (1970). Proc. Nat. Acad. Sci. USA. 67, 1248.
14. Jones, A.J. and Kuchel, P.W. (1980). Clin. Chim. Acta. 104, 77.
15. Young, J.D. and Ellory, J.C. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.) pp.301-325, Academic Press, New York.
16. Adibi, S.A. and Morse, E.L. (1971). J. Clin. Invest. 50, 2266.