Amino Acid Transport in Normal and Glutathione-Deficient Sheep Erythrocytes

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1. Uptake rates for 23 amino acids were measured for both normal (high-GSH) and GSH-deficient (low-GSH) erythrocytes from Finnish Landrace sheep. 2. Compared with high-GSH cells, low-GSH cells had a markedly diminished permeability to D-alanine, L-alanine, α -amino-*n*-butyrate, valine, cysteine, serine, threonine, asparagine, lysine and ornithine. Smaller differences were observed for glycine and proline, whereas uptake of the other amino acids was not significantly different in the two cell types. 3. High-GSH cells showed a 30-fold stereoselectivity for L-alanine as against D-alanine uptake, whereas low-GSH cells gave identical slow rates for both isomers. 4. Lysine uptake was consistent with a single component conforming to simple Michaelis-Menten kinetics with an apparent K_m of 110 mM for both cell types. 5. L-Alanine uptake by high-GSH cells also conformed to simple Michaelis-Menten kinetics (apparent K_m 17 mM). Low-GSH cells gave a linear relationship for this amino acid. 6. The apparent K_m for L-alanine uptake by high-GSH cells was markedly influenced by temperature. 7. No Na^+ dependence of glycine, L-alanine, leucine, phenylalanine or lysine uptake by high-GSH cells could be demonstrated. 8. Lysine uptake by high-GSH cells was markedly inhibited by L-alanine and α -amino-*n*-butyrate but not by D-alanine, and lysine significantly inhibited L-alanine uptake. 9. It is concluded that only one transport system is defective in low-GSH erythrocytes.

Congenital GSH deficiency in the erythrocytes of Finnish Landrace sheep is associated with high intracellular concentrations of certain amino acids, particularly ornithine and lysine (Tucker & Kilgour, 1970; Ellory *et al.*, 1972). We demonstrated that these cells show a markedly diminished uptake of cysteine, α -amino-*n*-butyrate and lysine (Young *et al.*, 1975). The decreased amino acid permeability is not a consequence of low intracellular GSH or high ornithine and lysine concentrations, but rather reflects a membrane-transport defect. The purpose of the present investigation was to establish the extent of this transport lesion by measuring the relative uptake rates of a series of natural amino acids by both normal (high-GSH) and GSH-deficient (low-GSH) erythrocytes.

Materials and Methods

Whole blood from Finnish Landrace sheep was collected by jugular venepuncture into heparinized vacuum containers. Animals were classified as to GSH type on the basis of erythrocyte GSH concentrations and amino acid analyses (Tucker & Kilgour, 1970; Ellory *et al.*, 1972). Blood samples were

screened by the Brilliant Cresyl Blue method (Dacie & Lewis, 1966), to ensure the absence of reticulocytes.

Materials

^{14}C -labelled amino acids were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All were uniformly labelled except histidine (ring-2- ^{14}C -labelled) and methionine (methyl- ^{14}C -labelled). DL- α -Amino-*n*-[1- ^{14}C]butyrate was obtained from Tracerlab, Boston, Mass., U.S.A.

Amino acid-uptake studies

Erythrocytes were washed three times with 20 vol. of a medium containing 106 mM-MgCl₂ and 10 mM-Tris/HCl (pH 7.5 at 20°C) and once in an incubation medium containing 135 mM-NaCl, 5 mM-KCl, 15 mM-Tris/HCl (pH 7.1 at 37°C), 3.1 mM-MgCl₂, 0.1 mM-EDTA and 5 mM-glucose. The buffy coat was discarded. The incubation medium for the temperature-dependence studies contained 15 mM-sodium phosphate (pH 7.1) in place of Tris/HCl. Choline replaced Na^+ in the Na^+ -dependence experiments.

Amino acid uptake was measured by mixing 0.5 ml of prewarmed washed erythrocytes with 0.5 ml of

incubation medium (at 37°C) containing the appropriate ^{14}C -labelled amino acid. The final haematocrit was approximately 10% with 0.05–0.40 μCi of ^{14}C -labelled amino acid/incubation. Iso-osmolality at various amino acid concentrations was maintained by adjusting the concentration of NaCl in the medium. Incubations containing cysteine also included dithiothreitol at a final concentration of 10 mM to prevent cysteine oxidation. It has been established that dithiothreitol does not influence amino acid uptake (Young *et al.*, 1975). At pre-determined time-intervals (5 min–2 h) incubations were stopped by rapidly washing the cells four times with 10 vol. of ice-cold MgCl_2 medium by using an Eppendorf 3200 microcentrifuge (10 s, 15000g). The packed cells were lysed in 0.5 ml of aq. 0.5% (v/v) Triton X-100 and 0.5 ml of 33% (w/v) trichloroacetic acid was added. The precipitate was removed by centrifugation (30 s, 15000g), and a sample (0.9 ml) of the supernatant transferred to 7 ml of Unisolve (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and counted for radioactivity in a β -scintillation spectrometer with quench correction.

Determination of kinetic constants

Where appropriate K_m and $V_{\max}$ values were determined from plots of s/v against s , fitting the best straight line to the points by eye.

Results

Time-course of amino acid uptake

The time-course of uptake of the 23 amino acids studied was established in a series of preliminary experiments. It was apparent that the uptake of certain amino acids was much faster than others. The incubation time for individual amino acids was therefore adjusted such that the uptake at all concentrations used was linear throughout the incubation period. The linearity of lysine and cysteine uptake (both 0.2 mM) by high- and low-GSH cells are illustrated in Figs. 1(a) and 1(b).

Amino acid uptake by high- and low-GSH erythrocytes

The initial rates of uptake of 23 naturally occurring amino acids by high- and low-GSH erythrocytes are presented in Table 1. High-GSH cells were permeable to all amino acids tested except for glutamate and aspartate. Alanine, α -amino-n-butyrate and cysteine gave the largest uptake rates, and glutamine and asparagine the lowest. The uptake of L-alanine was 30-fold greater than that of D-alanine.

Compared with high-GSH cells, low-GSH cells had a markedly diminished permeability to D-alanine, L-alanine, α -amino-n-butyrate, valine, cysteine, serine, threonine, lysine, ornithine and asparagine. Glycine and proline showed smaller, but still significant, differences in uptake between the two cell types. Uptake of the remaining amino acids was not significantly different between high- and low-GSH cells.

Concentration-dependence of amino acid uptake

From the relationship between amino acid concentration and uptake rate, Winter & Christensen (1964) proposed that there were two components of neutral-amino acid uptake in human erythrocytes: a saturable system with a finite K_m value, and a non-saturable system. We measured the concentration-dependence of uptake of four of the amino

Fig. 1. Time-course of lysine (a) and cysteine (b) uptake by high- and low-GSH cells

●, High-GSH cells, left-hand ordinate; ○, low-GSH cells, right-hand ordinate. The lysine and cysteine concentrations were both 0.2 mM. The cysteine incubation contained 10 mM-dithiothreitol.

Table 1. Uptake of amino acids by high- and low-GSH erythrocytes

Results are means $\pm$ S.E.M. for four animals of each type. The amino acid concentration was 0.2 mM; incubation times varied from 5 to 60 min as appropriate. N.S., Not significant.

Amino acid	Amino acid uptake (μ mol/h per litre of cells)		P	Ratio (high GSH)/ (low GSH)
	High GSH	Low GSH		
Glycine	5.98 $\pm$ 0.26	3.23 $\pm$ 0.34	<0.002	1.9
L-Alanine	154.4 $\pm$ 6.6	2.1 $\pm$ 0.4	<0.001	71.4
D-Alanine	5.06 $\pm$ 0.13	1.69 $\pm$ 0.01	<0.001	3.0
α -Amino- <i>n</i> -butyrate	698 $\pm$ 69	55 $\pm$ 11	<0.001	12.7
Valine	33.5 $\pm$ 0.5	9.8 $\pm$ 4.9	<0.01	3.4
Leucine	14.4 $\pm$ 1.4	16.4 $\pm$ 3.2	N.S.	
Isoleucine	12.6 $\pm$ 1.5	15.4 $\pm$ 3.4	N.S.	
Cysteine	229 $\pm$ 22	35 $\pm$ 3	<0.001	6.5
Serine	23.6 $\pm$ 1.9	3.0 $\pm$ 0.6	<0.001	7.9
Threonine	9.80 $\pm$ 0.55	3.32 $\pm$ 1.15	<0.01	3.0
Methionine	8.99 $\pm$ 1.37	10.80 $\pm$ 3.5	N.S.	
Cystine	8.63 $\pm$ 0.49	9.78 $\pm$ 0.22	N.S.	
Ornithine	16.1 $\pm$ 1.1	3.9 $\pm$ 1.0	<0.001	4.1
Lysine	15.0 $\pm$ 1.0	4.6 $\pm$ 1.0	<0.001	3.3
Arginine	4.86 $\pm$ 0.60	4.04 $\pm$ 0.97	N.S.	
Asparagine	6.55 $\pm$ 0.37	2.66 $\pm$ 0.71	<0.01	2.5
Glutamine	1.76 $\pm$ 0.40	2.65 $\pm$ 1.06	N.S.	
Phenylalanine	27.6 $\pm$ 2.2	29.0 $\pm$ 3.6	N.S.	
Tyrosine	28.7 $\pm$ 10.5	44.4 $\pm$ 23.2	N.S.	
Proline	7.04 $\pm$ 0.26	4.76 $\pm$ 0.65	<0.05	1.5
Histidine	8.77 $\pm$ 1.90	11.10 $\pm$ 4.7	N.S.	
Aspartate	<0.1	<0.1		
Glutamate	<0.1	<0.1		

acids (D-alanine, L-alanine, cysteine and lysine) which showed significant differences in uptake rates between high- and low-GSH erythrocytes. Lysine uptake was investigated over the concentration range 1–100 mM and was consistent with a single component conforming to simple Michaelis–Menten kinetics with an apparent K_m value of 111 mM for both high-GSH and low-GSH cell types, although V_{max} differed by a factor of 10 (Figs. 2a and 2b). The uptakes of D-alanine and L-alanine measured over the concentration range 0.5–9.0 mM for both high- and low-GSH animals were approximately linear for both cell types (Figs. 3a and 3b). L-Alanine uptake by high- and low-GSH cells was also investigated over the concentration range 0.75–20 mM (Fig. 4). For high-GSH cells the data were consistent with a single component conforming to simple Michaelis–Menten kinetics (apparent K_m 16.8 mM). This contrasted markedly with the linear relationship for low-GSH cells. The uptake of cysteine over the concentration range 0.5–9.0 mM was slightly non-linear in both cell types (Fig. 5).

Fig. 2. Concentration-dependence of lysine uptake by high- and low-GSH cells

(a) Concentration range 1–10 mM; (b) concentration range 10–100 mM. ●, High-GSH cells, left-hand ordinate; ○, low-GSH cells, right-hand ordinate. Curves in (b) were fitted as $v = 17.1/[1 + (111/s)]$ (high GSH) or $= 1.75/[1 + (111/s)]$ (low GSH), where v is the uptake in mmol/h per litre of cells and s is the concentration (mM).

Temperature-dependence of amino acid uptake

Hoare (1972), working with human erythrocytes, observed a dramatic fall in the apparent K_m value for leucine transport as the temperature was lowered. Figs. 6(a) and 6(b) demonstrate the effect of temperature on the kinetic parameters of L-alanine uptake by high-GSH cells. Decreasing the incubation temperature from 40° to 10°C resulted in a fivefold decrease in apparent K_m value and a 160-fold fall in V_{max} .

Fig. 3. Concentration-dependence of L-alanine (a) and D-alanine (b) uptake by high- and low-GSH cells

●, Low-GSH cells; ○, high-GSH cells.

Na⁺-dependence of amino acid uptake

Amino acid-transport systems in a variety of tissues have been shown to be Na⁺-dependent (e.g. see Christensen, 1969). Na⁺-dependent uptake has been demonstrated for some amino acids in human, rabbit and rat reticulocytes (Winter & Christensen, 1965; Yunis & Arimura, 1965; Antonioli & Christensen, 1969) and an Na⁺-dependent uptake of glycine and alanine has been claimed for mature human erythrocytes (Yunis & Arimura, 1965). Table 2 shows the uptake of five different amino acids by high-GSH erythrocytes in the presence and absence of Na⁺. No Na⁺ dependence was demonstrable. Lysine uptake was measured at two concentrations (0.1 and 10 mM) in case there was a Na⁺-dependent system with a low affinity. Again no Na⁺ effect was observed.

Fig. 4. Concentration-dependence of L-alanine uptake by high- and low-GSH cells

●, High-GSH cells, left-hand ordinate; ○, low-GSH cells, right-hand ordinate.

Fig. 5. Concentration-dependence of cysteine uptake by high- and low-GSH cells

●, High-GSH cells, left-hand ordinate; ○, low-GSH cells right-hand ordinate. The incubation medium contained 10 mM-dithiothreitol.

Effects of D-alanine, L-alanine and α-amino-n-butyrate on lysine uptake

The uptake of both neutral (smaller than leucine) and dibasic amino acids is decreased in low-GSH cells. To test whether these amino acids share a common transport system, the uptake of lysine (0.2 mM) by high-GSH cells was measured in the presence of various concentrations (10–50 mM) of D-alanine, L-alanine and α-amino-n-butyrate (Fig. 7). L-Alanine and α-amino-n-butyrate, but not D-alanine,

Fig. 6. Effect of temperature on L-alanine uptake by high-GSH cells

(a) Concentration dependence: ●, at 40°C, left-hand ordinate; ○, at 11°C, right-hand ordinate. (b) Effect of temperature on K_m and V_{max} values. ●, K_m ; ○, V_{max} .

Table 2. Na^+ -dependence of amino acid uptake by high-GSH erythrocytes

Values are means of duplicates.

Amino acid	Concn. (mm)	Amino acid uptake (μmol/h per litre of cells)	
		+Na ⁺	-Na ⁺
Lysine	0.1	14.7	13.9
Lysine	10.0	1510	1550
Glycine	0.2	13.0	13.7
Alanine	0.2	144	140
Leucine	0.2	24.6	26.2
Phenylalanine	0.2	48.9	47.5

Fig. 7. Effects of D-alanine, L-alanine and α-amino-n-butyrate on lysine uptake by high-GSH cells

●, D-Alanine; ■, L-alanine; ▲, α-amino-n-butyrate. The lysine concentration was 0.2 mM.

markedly inhibited lysine uptake. In the complementary experiment 50 mM-lysine inhibited L-alanine uptake (0.2 mM) by 20%.

Discussion

The present results confirm and extend previous findings (Young *et al.*, 1975) of differences in amino acid uptake by high- and low-GSH erythrocytes from Finnish Landrace sheep. Of the 23 amino acids studied, only 10 amino acids, namely D-alanine, L-alanine, α-amino-n-butyrate, valine, cysteine, serine, threonine, asparagine, lysine and ornithine showed marked differences in uptake rate between the two cell types.

In high-GSH cells the highest uptake rates were given by L-alanine, α-amino-n-butyrate and cysteine. These amino acids, together with serine, gave the largest differences between the two cell types, suggesting the presence in the high-GSH cell of a transport system for these amino acids which is defective in the low-GSH cell. The comparison of D-alanine and L-alanine demonstrates that this system ('the cysteine system') is specific for the L series. It is striking that whereas L-alanine uptake was 30-fold greater than D-alanine in the high-GSH cell, the two amino acids entered the low-GSH cell at the same slow rate. It is probable therefore that the cysteine-transport system is completely absent from the low-GSH cell and that the residual L-alanine uptake by these cells occurs by a different route which is not stereospecific. This is confirmed by the concentration dependence of L-alanine uptake by high- and low-GSH cells. Whereas L-alanine uptake

by high-GSH cells conformed to simple Michaelis-Menten kinetics, the residual L-alanine uptake by low-GSH cells was linear over the same concentration range. Of the remaining amino acids which showed different uptake rates in the two cell types, valine, threonine and possibly asparagine are probably also transported by the cysteine system. Approximately 50% of the glycine and 30% of the proline uptake may occur by this route. However, the cysteine system does not seem to be involved in the uptake of either long-chain aliphatic or aromatic amino acids.

Ornithine and lysine are the only remaining amino acids that gave differences in uptake between high- and low-GSH cells. It is difficult to reconcile these two basic amino acids with the group of neutral, structurally similar amino acids that share the cysteine system. The decreased ornithine and lysine uptake by low-GSH cells is not a consequence of the elevated intracellular concentrations of these amino acids, since high-GSH cells preloaded with lysine (intracellular concentration 3.0 mM) show normal lysine uptake rates (results not shown). It is therefore possible that cysteine and ornithine and lysine enter the sheep erythrocyte by two separate systems, both of which are defective in low-GSH cells. However, lysine uptake by high-GSH cells was markedly inhibited by L-alanine and α -amino-n-butyrate, but not by D-alanine, and L-alanine uptake was significantly inhibited by lysine, suggesting that these amino acids share a common transport system. It is therefore concluded that only one transport system (the cysteine system) is defective in low-GSH erythrocytes.

The amino acid specificity of the proposed cysteine-transport system is unlike that of previously described mammalian erythrocyte transport systems (Winter & Christensen, 1964, 1965; Antonioli & Christensen, 1969; Gardner & Levy, 1972). However, in this respect it does resemble the alanine-serine-cysteine (ASC) system defined by Christensen and his co-workers (Christensen, 1969, 1975). The alanine-serine-cysteine system, which has been found in the

rabbit reticulocyte, but not in mature mammalian erythrocytes, also shares the cysteine system's stereospecificity. However, the alanine-serine-cysteine system, unlike the present system, is Na^+ -dependent, and in rabbit reticulocytes appears largely limited to transport by exchange. This cannot be the case for the cysteine system, since washed high-GSH cells contain very low amino acid concentrations (Ellory *et al.*, 1972). We conclude that the present system differs from those previously described for mammalian reticulocytes and mature erythrocytes. It is likely that the physiological role of this system involves the transport of cysteine for the intracellular biosynthesis of GSH. Its absence results in a markedly diminished erythrocyte GSH concentration (approximately 30% of normal).

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