Biochem. J. (1991) 277, 565–568 (Printed in Great Britain)

Use of membrane vesicles to estimate the numbers of system y^+ and system L amino acid transporters in human erythrocytes

Chung-Ming TSE,* Daron A. FINCHAM,† J. Clive ELLORY‡ and James D. YOUNG§‖

*Department of Medicine, G. I. Division, The Johns Hopkins University School of Medicine, 725 Wolfe Street, Hungterian #503, Baltimore, MD 21205, U.S.A., †School of Applied Sciences, Wolverhampton Polytechnic, Wulfruna Street, Wolverhampton WVI 1SB, U.K., ‡University Laboratory of Physiology, South Parks Road, Oxford OX1 3PT, U.K., and §Department of Physiology, 7-55 Medical Sciences Building, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

We have used equilibrium values for L-leucine and L-lysine uptake by right-side-out vesicles to estimate the membrane abundance (sites/cell) of Na^+-dependent amino acid transport systems L and y^+ in human erythrocytes. All of the intravesicular space was accessible to L-leucine, as judged by comparisons with uridine uptake via the equilibrative nucleoside transporter (10^4 sites/cell). In contrast, only 28% of the total intravesicular space was accessible to L-lysine uptake via system y^+. Since human erythrocyte membranes generate an average of ~1000 vesicles/cell, these data provide evidence that system L is a relatively high-abundance membrane transport protein in human erythrocytes, while system y^+ is present in smaller amounts (~300 copies/cell). Calculated turnover numbers for L-lysine transport by system y^+ at 37 °C are 24 s^{-1} for zero-*trans* influx and 150 s^{-1} for equilibrium-exchange influx.

INTRODUCTION

In marked contrast with most other classes of plasma membrane transport systems, no specific high-affinity inhibitors of mammalian amino acid transporters are known, and systems have been identified and characterized entirely on the basis of substrate specificity and cation/anion requirements [1–3]. This lack of suitable covalent or reversibly binding probes has hampered molecular studies of amino acid transport proteins [4–9] and has provided an obstacle to obtaining quantitative data on the membrane abundance (sites/cell) of such systems in different cell types and tissues. This latter information is required for a full kinetic understanding of amino acid permeation mechanisms, and is also a necessary prerequisite for isolation and purification studies.

Erythrocytes from different mammalian and other vertebrate species express a diverse array of different amino acid transporters, making this cell type an ideal model system for amino acid permeation studies [10,11]. Two equilibrative amino transporters present in human erythrocytes are system L, which is selective for large hydrophobic amino acids such as leucine and phenylalanine [12–15], and system y^+, which is specific for the dibasic amino acids lysine, ornithine and arginine [14,16,17]. We report here that plasma membranes prepared from human erythrocytes retain system L and system y^+ activity after vesiculation into right-side-out membrane vesicles. Based upon a novel approach developed originally by Lew *et al.* [18] for studies of the human erythrocyte Ca^{2+}-activated K^+ channel, we have used vesicle equilibration volumes for leucine and lysine uptake to provide, for the first time, information on the numbers of system y^+ and system L transporters present in human erythocytes.

MATERIALS AND METHODS

Vesicle preparation

Freshly drawn heparinized human blood was washed three times in 150 mM-NaCl/5 mM-sodium phosphate (pH 8.0). The buffy coat was discarded. To prepare erythrocyte ghosts, packed cells (2 ml) were haemolysed in 40 ml of ice-cold 5 mM-sodium phosphate (pH 8.0) and the membranes were washed three times by repeated centrifugation (30000 *g* for 10 min) and resuspension in 40 ml of the same solution. The final haemoglobin-free membrane pellet was resuspended in 2 ml of 5 mM-sodium phosphate (pH 8.0).

Right-side-out membrane vesicles were prepared by the method of Steck [19], as modified by Cohen & Solomon [20] and Tse *et al.* [21]. A 2 ml portion of ghosts prepared as described above was diluted to 50 ml with 0.5 mM-sodium phosphate (pH 8.0) and left on ice for 30 min. At the end of this period, 0.1 mM-$MgCl_2$ (final) was added to the suspension. Membranes were then pelleted by centrifugation (30000 *g* for 1 h) and the pellet was kept at 4 °C overnight to promote vesiculation. After overnight incubation, the membrane pellet was resuspended to 2 ml in 0.1 mM-$MgCl_2$/0.5 mM-sodium phosphate (pH 8.0) and homogenized by passing five times through a no. 27 gauge needle. The suspension was then layered on to 3 ml of Dextran T-70 barrier solution [45% (w/v) Dextran T-70, 0.1 mM-$MgSO_4$, 0.5 mM-sodium phosphate, pH 8.0] in a 5 ml Beckman SW 50.1 centrifuge tube and centrifuged at 30000 *g* for 4 h. Sealed right-side-out membrane vesicles were harvested from the barrier interface, washed twice with 5 mM-sodium phosphate (pH 8.0) and resuspended and stored at 4 °C in the same buffer at 2–4 mg of protein/ml.

Right-side-out vesicles were determined to be > 98% pure by glyceraldehyde-3-phosphate dehydrogenase assay as described by Steck [19].

Transport studies

Vesicle transport experiments were performed at 25 °C by Millipore filtration techniques [21–23].

Influx experiments. Amino acid uptake was initiated by addition of radioactive permeant (L-[U-^{14}C]leucine, L-[U-^{14}C]lysine monohydrochloride or L-[U-^{14}C]glutamic acid; 0.4 mM, 4 μCi/ml) in 5 mM-sodium phosphate (pH 8.0) to an equal volume of membrane vesicle suspension (1.5 mg of protein/ml). At ap-

Abbreviation used: NBTGR, nitrobenzylthioguanosine.

‖ To whom correspondence and reprint requests should be sent.

propriate times thereafter, uptake was terminated by taking 30 μl aliquots of the incubation mixture into 2 ml of ice-cold stopping solution (250 mM-NaCl, 5 mM-sodium phosphate, 20 μM-phloretin, pH 8.0), followed by immediate filtration under vacuum using Millipore HAWP 0.45 μm filters which had been prewashed with 2 ml of stopping solution. Inclusion of salt in the stopping solution was to increase the retention of vesicles on the filter, while phloretin is a system L inhibitor. Filters were washed rapidly with 4 x 2 ml of ice-cold stopping solution and transferred into counting minivials containing 4 ml of scintillation fluid for radioactivity determination.

Uridine-trapping space was determined by incubating [2-^{14}C]uridine with a membrane vesicle suspension [0.2 mM, 2 μCi/ml (final concentrations) at a protein concentration of 0.75 mg/ml] for 5 min, during which time all of the vesicles would have equilibrated with uridine [21]. Then, 30 μl of incubation mixture was added to 2 ml of stopping solution [250 mM-NaCl, 5 mM-sodium phosphate, 20 μM-nitrobenzylthioguanosine (NBTGR), pH 8.0] and processed as described above.

Blank values were obtained by processing samples in ice-cold uridine, or amino acid solution was mixed with ice-cold membrane vesicle suspension pretreated with 20 μM-NBTGR or phloretin and immediately diluted with stopping solution. Vesicle uptake was calculated after subtraction of these blanks. No intravesicular radioactivity was lost from vesicles during washing (~15 s), and filter retention of vesicle radioactivity was confirmed to be directly proportional to vesicle density in the range 5–30 μg of protein/assay.

Efflux experiments. Membrane vesicles at a protein concentration of 1.5 mg/ml were equilibrated with 0.2 mM radioactive permeant (2 μCi/ml, final concentration) for 2 h at 25 °C. To initiate efflux, 30 μl of incubation mixture was added to 4 ml of 5 mM-sodium phosphate (pH 8.0) at 25 °C. By this means, the vesicles were diluted 130-fold with medium such that the extravesicular permeant concentration was negligible. At the appropriate time thereafter, 1 ml of ice-cold 1.25 M-NaCl/5 mM-sodium phosphate (pH 8.0) containing 100 μM-phloretin (for amino acid experiments) or 20 μM-NBTGR (for uridine experiments) were added to terminate the reaction, followed by immediate filtration under vacuum as described for influx experiments. Filters were washed rapidly with 4 x 2 ml of ice-cold stopping solution (250 mM-NaCl, 5 mM-sodium phosphate, pH 8.0) with 20 μM-phloretin or 20 μM-NBTGR and then transferred to counting minivials with 4 ml of scintillation fluid for radioactivity determination.

Materials

Radiolabelled amino acids and uridine were obtained from Amersham International, Amersham, Bucks., U.K. Other reagents were obtained as follows: non-radioactive amino acids, uridine, NBTGR and phloretin were from Sigma, Kingston-upon-Thames, Surrey, U.K., and Dextran T-70 was from Pharmacia Fine Chemicals, Hounslow, Middlesex, U.K. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Vesiculation of human erythrocyte membranes under standard conditions generates 500–1500 vesicles per cell [18,19]. This is substantially less than the numbers of copies per erythrocyte of several well-characterized erythrocyte membrane transport systems, such as the erythrocyte anion (band 3), glucose and nucleoside transporters (approx. 10^6, 2×10^5 and 10^4 copies per cell respectively) [24–26]. Human erythrocyte membrane vesicles will therefore contain multiple copies of each of these three transporters and all of the intravesicular space will be accessible to the permeants (e.g. glucose, uridine). This is not the case for low-abundance transport systems such as the Na^+ pump (100–200 sites per cell) [27], where membrane vesiculation yields a mixture of transport-competent and transporter-deficient vesicles [18]. Under these circumstances, only a fraction of the total intravesicular space is accessible to permeant. Using human erythrocyte inside-out membrane vesicles, Lew *et al.* [18] demonstrated that the percentage of the total intravesicular space accessible via the Ca^{2+}-activated K^+ channel (^{86}Rb efflux activated by external Ca^{2+}) was similar to that available to the Na^+ pump (^{86}Rb efflux in the presence of external Na^+ and ATP). On the basis of this result, it was concluded that the two cation transport systems have similar numbers of copies per cell. The objective of the present study was to use a similar approach to investigate the membrane abundance of two human erythrocyte amino acid transporters, the neutral amino acid transport sytem L and the dibasic amino acid carrier, system y^+.

Fig. 1 shows a representative time-course experiment comparing the uptake (0.2 mM concentration, 25 °C) of L-lysine, L-leucine, L-glutamate and uridine by human erythrocyte right-side-out membrane vesicles. L-Lysine and L-leucine were selected as representative system y^+ and system L permeants respectively, while L-glutamate, which is largely impermeant in intact human erythrocytes [14], was a control for vesicle integrity. Uridine, a nucleoside transporter permeant [26], was included in the experiment to report the total intravesicular space of the preparation. Uptake of both L-lysine and L-leucine proceeded rapidly during the first 2 min of incubation. For L-leucine, the 5 min equilibration value was identical to that for uridine and corresponded to an uptake of 1.37 nmol/mg of protein. Similar values for uridine uptake have been obtained by us previously for both inside-out and right-side-out human erythrocyte membrane vesicles [21]. To exclude the possibility of amino acid binding to vesicles, rather than transport, we equilibrated vesicles with 0.2 mM radiolabelled L-leucine (5 min incubation), after which 10 mM non-radioactive L-leucine was added to the suspension. This resulted in the predicted rapid loss of radioactivity from the vesicles (79 % in 30 s) and was followed by a slow re-equilibration of tracer with the intravesicular space. In other control experiments, uptake of L-leucine by vesicles (0.2 mM, 10 s incubation)

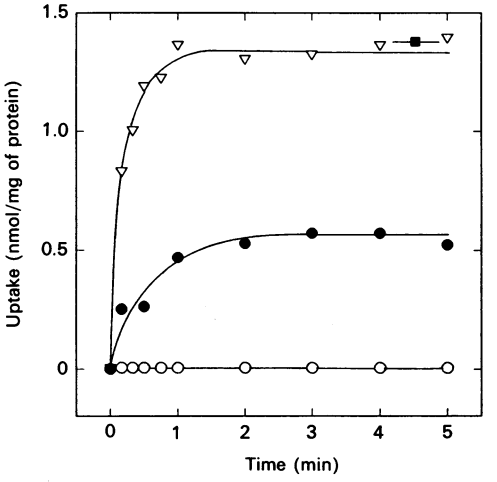

Fig. 1. Uptake of amino acids and uridine by human erythrocyte right-side-out membrane vesicles

Transport of 0.2 mM radiolabelled L-leucine (▽), L-lysine (●), L-glutamate (○) and uridine (■) was measured at 25 °C as described in the text. Individual data points are means of triplicate determinations.

Table 1. Vesicle equilibration volumes for uridine, L-lysine and L-leucine

Right-side-out membrane vesicles were incubated with 0.2 mM radioactive permeant at 25 °C as described in the text. Numbers of experiments are given in parentheses. Results are means ± S.E.M.

Substrate	Uptake at 5 min (nmol/mg of protein)	Equilibration (%)
Uridine (7)	1.40 ± 0.04	100
L-Leucine (4)	1.42 ± 0.08	100 ± 5
L-Lysine (4)	0.39 ± 0.06	28 ± 4

Table 2. Specificity of L-lysine uptake by human erythrocyte right-side-out membrane vesicles

Uptake of 0.2 mM-L-[^{14}C]lysine was measured at 25 °C as described in the text in the absence and in the presence of competing non-radioactive amino acids at a concentration of 2 mM. Permeant and non-radioactive amino acids were added to vesicles simultaneously. Values are expressed as percentages of control uptake in the absence of competing non-radioactive amino acid, and are means of triplicate determinations.

Amino acid	Uptake rate (% of control)
L-Lysine	7
L-Ornithine	26
L-Arginine	26
L-Glutamate	96
L-Alanine	92
L-Leucine	102

was confirmed to be highly temperature-dependent ($Q_{10} \simeq 4$) and sensitive to inhibition by the system L permeant L-phenylalanine (84% inhibition, 15 mM-L-phenylalanine). In contrast to L-leucine and uridine, L-lysine uptake reached a plateau at a substantially lower value of 0.53 nmol/mg of protein, an L-lysine/(L-leucine, uridine) equilibration ratio of 0.39. Results from four separate vesicle preparations gave a mean lysine/(leucine, uridine) ratio of 0.28 ± 0.04 (5 min incubation, Table 1). In contrast with results with these three permeants, vesicles showed no significant uptake of L-glutamate after 5 min (Fig. 1).

To establish that the observed uptake of L-lysine by vesicles was mediated by system y+, the initial rate of L-lysine uptake (0.2 mM, 15 s incubation) was measured in the presence of a series of competing non-radioactive amino acids (2.0 mM). As shown in Table 2, radiolabelled L-lysine uptake was substantially inhibited by non-radioactive L-lysine, L-ornithine and L-arginine, whereas L-leucine, L-alanine and L-glutamate had little effect. In addition to transmembrane permeation via system y+, L-lysine is transported slowly and with low affinity by system L [14]. Also, intact human erythrocytes exhibit a measurable rate of lysine diffusion across the lipid bilayer [14]. These secondary permeation pathways allowed lysine to equilibrate fully with the intravesicular space after 2 h at 25 °C. Under these prolonged incubation conditions, L-glutamate reached one-third equilibration with the intravesicular space. In intact cells, this amino acid is accepted as an 'accidental' substrate by the band 3 anion-exchange transporter, and some diffusion across the lipid bilayer also occurs [28].

Based upon these results, we conclude that system L has access to the full intravesicular space, but that system y+, like the Na+ pump and the Ca^{2+}-activated K^+ channel [18], is present in some vesicles only. This interpretation is consistent with results from the vesicle efflux experiment presented in Fig. 2. This experiment demonstrates that vesicles pre-loaded with radioactive uridine and L-leucine (0.2 mM; 2 h at 25 °C) lost all of their intravesicular permeant within 1 min, the rate of uridine efflux being ~6-fold greater than that for leucine. Phloretin, at the concentration used in the transport assay stopping solution (20 μM), decreased the rate of L-leucine efflux from vesicles 4-fold (results not shown). In contrast with these efflux profiles, but as expected from the L-lysine uptake data in Fig. 1 and Tabler 1, only 27% of the previously accumulated radioactivite L-lysine was readily lost from the vesicles.

Quantitatively, these results indicate that system L is a relatively high-abundance transport protein in human erythrocyte membranes (≥ 500 copies per cell), while system y+ is present in smaller amounts. On the assumption that there are no interactions between individual transporters, the distribution of transporters in vesicles should follow the Poisson distribution:

$$f(n) = \frac{x^n e^{-x}}{n!} \quad (1)$$

where $f(n)$ is the fraction of vesicles with n transporters per vesicle and x is the average number of transporters per vesicle in the whole vesicle population. Consequently, the average number of transporters per vesicle can be calculated from the fraction of vesicles without transporter, which is equal to the portion of vesicular volume that does not equilbrate with amino acid.

According to eqn. (1):

$$f(o) = e^{-x}$$

or:

$$\ln f(o) = -x$$

For lysine transport via system y+, $f(o) = (1-0.28)$ or 0.72, and hence $x = 0.33$. Assuming that 500–1500 vesicles are produced per cell [18,19], there are estimated to be between 170 and 500 copies of the lysine transporter per cell.

Previously, we have established that the $V_{max.}$ for system y+-mediated uptake of L-lysine by human erythrocytes at 37 °C is 0.49 mmol/h per litre of cells (K_m 0.068 mM) [14]. Under true

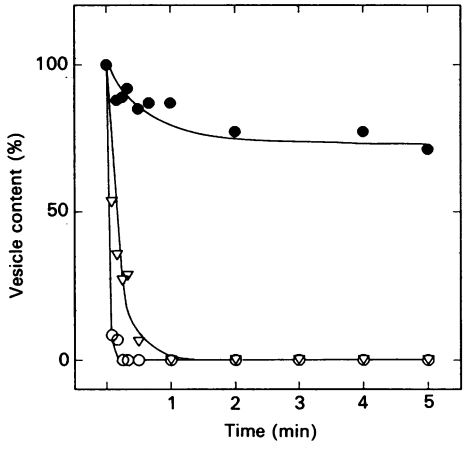

Fig. 2. Efflux of amino acids and uridine from human erythrocyte right-side-out membrane vesicles

Efflux of radiolabelled L-leucine (▽), L-lysine (●) and uridine (○) from vesicles preloaded with 0.2 mM-permeant was measured at 25 °C as described in the text. Values are means of triplicate determinations.

zero-*trans* influx conditions in cells depleted of free intracellular amino acids, this is decreased to 0.22 mmol/h per litre of cells (K_m 0.057 mM), while under equilibrium-exchange influx conditions the $V_{max.}$ is 1.37 mmol/h per litre of cells, with an apparent K_m value of 0.35 mM (C. M. Harvey & J. C. Ellory, unpublished work). By combining these $V_{max.}$ values with the estimated number of system y^+ transporters per cell (approx. 300), we can, for the first time, derive estimates of zero-*trans* and equilibrium-exchange turnover numbers for a mammalian amino acid transporter. $V_{max.}$ values of 0.22 and 1.37 mmol/h per litre of cells are equivalent to 7200 and 45000 molecules of amino acid transported/s per cell, giving rise to turnover numbers of approx. 24 and 150 s^{-1} for zero-*trans* influx and equilibrium-exchange influx respectively. These values are an order of magnitude lower than the corresponding turnover numbers for the erythrocyte glucose and nucleoside transporters [29], but are well within the range of turnover numbers calculated for other membrane transport systems [29].

It will be interesting to establish whether the relatively low turnover number of the erythrocyte lysine transporter compared with other erythrocyte facilitated-diffusion systems is a general property of all amino acid transporters or whether it is perhaps a consequence of the charged nature of the permeant. Although it is impossible to perform the same calculations for system L, since all vesicles showed transport, the present data can be used to predict a higher turnover number for system L. This is because, at a $V_{max.}$ of 120 mmol/h per litre of cells at 37 °C for zero-*trans* L-leucine influx (K_m 5.8 mM) [14], the abundance of system L in human erythrocytes would be equivalent to that of the glucose transporter, which comprises 5 % of the total integral membrane protein.

The conclusions drawn from the present study depend upon the assumption that system y^+ activity is stable and that no inactivation occurs during the procedures used to prepare vesicles. It is our experience that the transporter, in intact cells, retains activity and remains fully functional both during prolonged storage of blood or washed cells at 4 °C and during extended incubation at 37 °C. The possibility of transporter inactivation during membrane isolation and subsequent vesiculation is difficult to evaluate experimentally, but is unlikely in view of the mild nature of the conditions used and the fact that a wide range of other membrane transport systems (including amino acid transport system L) remain functional during these procedures.

This work was supported by the Royal Hong Kong Jockey Club, the University and Polytechnics Grants Committee, Hong Kong, and the Alberta Heritage Foundation for Medical Research. J.D.Y. is a Heritage Medical Scientist.

Received 8 March 1991/16 April 1991; accepted 3 May 1991

REFERENCES

1. Christensen, H. N. (1979) Adv. Enzymol. **49**, 41–101
2. Christensen, H. N. (1984) Biochim. Biophys. Acta **779**, 255–269
3. Kilberg, M. S. (ed.) (1986) Fed. Proc. Fed. Am. Soc. Exp. Biol. **45**, 2438–2454
4. Wright, E. M. & Peerce, B. E. (1984) J. Biol. Chem. **259**, 14993–14996
5. Fincham, D. A., Ellory, J. C. & Young, J. D. (1985) Biochem. Soc. Trans. **13**, 229
6. Ellory, J. C., Fincham, D. A. & Young, J. D. (1985) J. Physiol. (London) **369**, 154P
7. Radian, R., Bendahan, A. & Kanner, B. I. (1985) J. Biol. Chem. **261**, 15437–15441
8. McCormick, J. I. & Johnston, R. M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7877–7881
9. Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A. & Kanner, B. I. (1990) Science **249**, 1303–1306
10. Young, J. D. & Ellory, J. C. (1977) in Membrane Transport in Red Cells (Ellory, J. C. & Lew, V. L., eds.), pp. 301–325, Academic Press, London and New York
11. Harvey, C. M. & Ellory, J. C. (1989) Methods Enzymol. **173**, 122–160
12. Winter, C. G. & Christensen, H. N. (1964) J. Biol. Chem. **239**, 872–878
13. Hoare, D. G. (1972) J. Physiol. (London) **221**, 331–348
14. Young, J. D., Jones, S. E. M. & Ellory, J. C. (1980) Proc. R. Soc. London B. **209**, 355–375
15. Rosenberg, R. (1981) J. Membr. Biol. **62**, 79–93
16. Antonioli, J. A. & Christensen, H. N. (1969) J. Biol. Chem. **244**, 1505–1509
17. Gardner, J. D. & Levy, A. G. (1972) Metab. Clin. Exp. **21**, 413–431
18. Lew, V. L., Muallem, S. & Seymour, C. A. (1982) Nature (London) **296**, 742–744
19. Steck, T. L. (1974) in Methods in Membrane Biology (Korn, E. D., ed.), pp. 245–281, Plenum Press, New York
20. Cohen, C. M. & Solomon, A. K. (1976) J. Membr. Biol. **29**, 345–372
21. Tse, C. M., Wu, J. S. R. & Young, J. D. (1985) Biochim. Biophys. Acta **818**, 316–324
22. Macintyre, J. D. & Gunn, R. B. (1981) Biochim. Biophys. Acta **644**, 351–362
23. Seymour, C. A., Muallem, S. & Lew, V. L. (1982) in Red Cell Membranes: A Methodological Approach (Ellory, J. C. & Young, J. D., eds.), pp. 219–222, Academic Press, London and New York
24. Lin, S. & Spudich, J. A. (1974) J. Biol. Chem. **249**, 5778–5783
25. Wieth, J. O. (1979) J. Physiol. (London) **294**, 521–539
26. Jarvis, S. M. & Young, J. D. (1980) Biochem. J. **190**, 377–383
27. Lew, V. L., Muallem, S. & Seymour, C. A. (1982) Nature (London) **296**, 742–744
28. Fincham, D. A., Teoh, R., Mason, D. K. & Young, J. D. (1989) Biochem. Soc. Trans. **17**, 541–542
29. Stein, W. D. (1986) Transport and Diffusion across Cell Membranes, pp. 356–357, Academic Press, London and New York